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**AN *IN-VIVO* ASSESSMENT OF
ENDOTHELIAL FUNCTION AND
ARTERIAL WALL BIOMECHANICS
IN THE SUBGROUPS OF
SCLERODERMA**

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MASTER OF SURGERY

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Thesis Abstract

Scleroderma (systemic sclerosis; SSc) is a rare disorder, characterised by fibrosis predominantly affecting the skin and internal organs. The commonest SSc subgroups are limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc), together accounting for 90% of cases. Critical events in the vascular pathogenesis of both subgroups are *endothelial dysfunction* and altered *arterial biomechanics*, which together cause the increased vasomotor tone and heightened vasoconstricting potential seen in these patients.

The aim of this research was to examine two central hypotheses. First, that endothelial dysfunction can be demonstrated *in-vivo* in SSc subgroups, both in medium-sized muscular arteries and in the microcirculation. Second, that altered arterial wall biomechanics, manifesting as increased stiffness and abnormal viscoelasticity, can be demonstrated *in-vivo* in both medium-sized muscular arteries and large elastic arteries.

Flow-mediated dilatation of the brachial artery and arterial elasticity of the brachial and carotid arteries, were assessed *in-vivo* by high-resolution ultrasound coupled to echo-locked wall-tracking software, in groups of 20 lcSSc, 20 dcSSc and 20 healthy controls. Arterial viscoelasticity was assessed using applanation tonometry in 13 lcSSc, 14 dcSSc and 13 healthy controls. Microvascular endothelial function was studied *in-vivo* using laser Doppler iontophoresis, in 15 lcSSc and 15 dcSSc patients.

We concluded that: first, when compared to lcSSc, dcSSc manifests significantly impaired endothelial function in the brachial artery, but not in the microvasculature; second, that increased arterial stiffness is a feature of the brachial artery, and not confined to the carotid as previously thought; and third, viscoelasticity of the carotid artery, as demonstrated using a novel non-invasive technique is not significantly impaired.

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List of Abbreviations

ACA	Anti-centromere antibody
ACE	Angiotensin converting enzyme
ACR	American College of Rheumatology
AECA	Anti-endothelial cell antibodies
Aix	Augmentation index
ANA	Anti-nuclear antibody
ARIC	Atherosclerosis Risk in Communities
BK	Bradykinin
cbEGF	Calcium-binding epidermal growth factor
cGMP	cyclic guanosine-3',5-monophosphate
CMV	Cytomegalovirus
CPT	Cold pressor testing
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
dcSSc	Diffuse cutaneous systemic sclerosis
EC	Endothelial cells
ECM	Extra-cellular matrix
edNO	Endothelium-derived nitric oxide
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ET _A	ET-1 receptor A

ET _B	ET-1 receptor B
fbn1	Fibrillin-1
FMD	Flow-mediated dilatation
GTN	Glyceryl trinitrate
IL-1	Interleukin 1
iNOS	Inducible nitric oxide synthase
IVUS	Intravascular ultrasound
lcSSc	Limited cutaneous systemic sclerosis
LDI	Laser Doppler Iontophoresis
L-NMMA	L-arginine analogue <i>N</i> ^G -monomethyl-L-arginine
MBF	Myocardial blood flow
mRSS	Modified Rodnan skin score
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PAH	Pulmonary artery hypertension
PET	Positron emission tomography
PWA	Pulse-wave analysis
Scl-70	Anti-topoisomerase antibody
SMC	Smooth muscle cells
SNP	Single nucleotide polymorphisms
SNP	Sodium nitroprusside
SSc	Systemic sclerosis

TGF- β	Transforming growth factor- β
Tsk	Tight skin mice
TVRS	Total vascular risk score
VOP	Venous occlusion plethysmography
WTS	Wall track system
5-HT	5-hydroxy triptamine

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*This thesis is dedicated to my wife Jabin,
and to my children Zara and Rayan.*

I am also grateful to my parents for all their help and support.

Chapter One

Introduction

1.1 Introduction to “Systemic Sclerosis”

1.1a. A brief history

The original derivation of the word “scleroderma” comes from the Greek words *sclero* and *derma*, which together literally mean “hardskin”. It was probably first documented in the painting *Archangel Raphael and Bishop Francisco Domonte* by Murillo (1680). In it, Murillo depicts the bishop as having a notable tightening of the skin around the face and numerous small telangiectases on the face, lips, and hands; all classical cutaneous features of scleroderma (Figure 1.1). However, the Italian Carlo Cruzio is credited as the first modern physician to identify this condition in the year 1753¹.

Throughout the 18th and 19th centuries, our understanding of scleroderma grew steadily but slowly. For a long time it was considered to be a very rare, essentially cutaneous, progressive disorder that seemed to afflict women predominantly, and was widely acknowledged to be very difficult, if not impossible, to treat. In the mid 20th century, some physicians began to observe that not all patients with scleroderma presented in the same way; indeed some patients had markedly different patterns of skin hardening compared to others²⁻⁵. It was also observed that, as the disease progressed, some patients with scleroderma developed internal organ disease, typically affecting the heart, lungs, kidney, and gut, while others had a less aggressive course⁶⁻¹². As the multi-system nature of scleroderma became increasingly apparent, these physicians were prompted to re-designate the condition *systemic sclerosis (SSc)*^{2;7;13}.

Today, although the term “scleroderma” is still widely used, it is regarded as a generic term that can be used to represent any one of a closely related group of connective tissue disorders. These disorders are characterised by excessive fibrosis and

collagen deposition, affecting to various degrees the skin, internal organs and systemic vascular tree. Together, these disorders are called the *scleroderma spectrum*, and they are summarised in Table 1.1^{14;15}. The diseases within this spectrum range in severity from relatively mild dermal sclerosis, to severe progressive systemic sclerosis with multiple organ involvement, to pure vascular conditions such as Raynaud's phenomenon.

Figure 1.1 Murillo's painting of Archangel Raphael and Bishop Francisco Domonte. The Bishop, depicted in the lower right hand corner of the main image (A) and enlarged (B), is thought to demonstrate some of the typical cutaneous features of scleroderma – namely skin tightening and telangiectases around the skin of the hand and face.

A**B**

1.1b. Subgroups of SSc

Systemic sclerosis can be subdivided on clinical grounds into several closely-related but distinctive subgroups. The two main subgroups are limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc), and together they account for approximately 90% of SSc patients (60% and 30% respectively)¹⁶. They are distinguished primarily by pattern of skin involvement, but can also be distinguished by antibody profile, capillaroscopic findings, and internal organ involvement¹⁷. These two conditions, together with others, form part of the aforementioned scleroderma spectrum (Table 1.1).

Table 1.1 The scleroderma spectrum.

Disease type	Distribution
Scleroderma only	
Localised scleroderma	Skin involvement only
Plaque morphoea	< 4 areas of localised involvement
Generalised morphoea	> 4 areas or widespread disease
Linear scleroderma	Linear distribution, commonest childhood form
En coup de sabre	Scalp and facial lesions
Systemic sclerosis (SSc)	
Diffuse cutaneous systemic sclerosis	Proximal skin and internal organ disease
Limited cutaneous systemic sclerosis	Distal skin and internal organ disease
Intermediate cutaneous systemic sclerosis	Features of both limited and diffuse disease
Overlap syndromes	Often associated with other rheumatic diseases
Systemic sclerosis <i>sine</i> scleroderma	Internal organ disease but no skin involvement
Isolated Raynaud's phenomenon	
Primary	Quite common, negative antibody profile
Secondary	May be precursor to SSc, positive antibodies

1.1c. Epidemiology

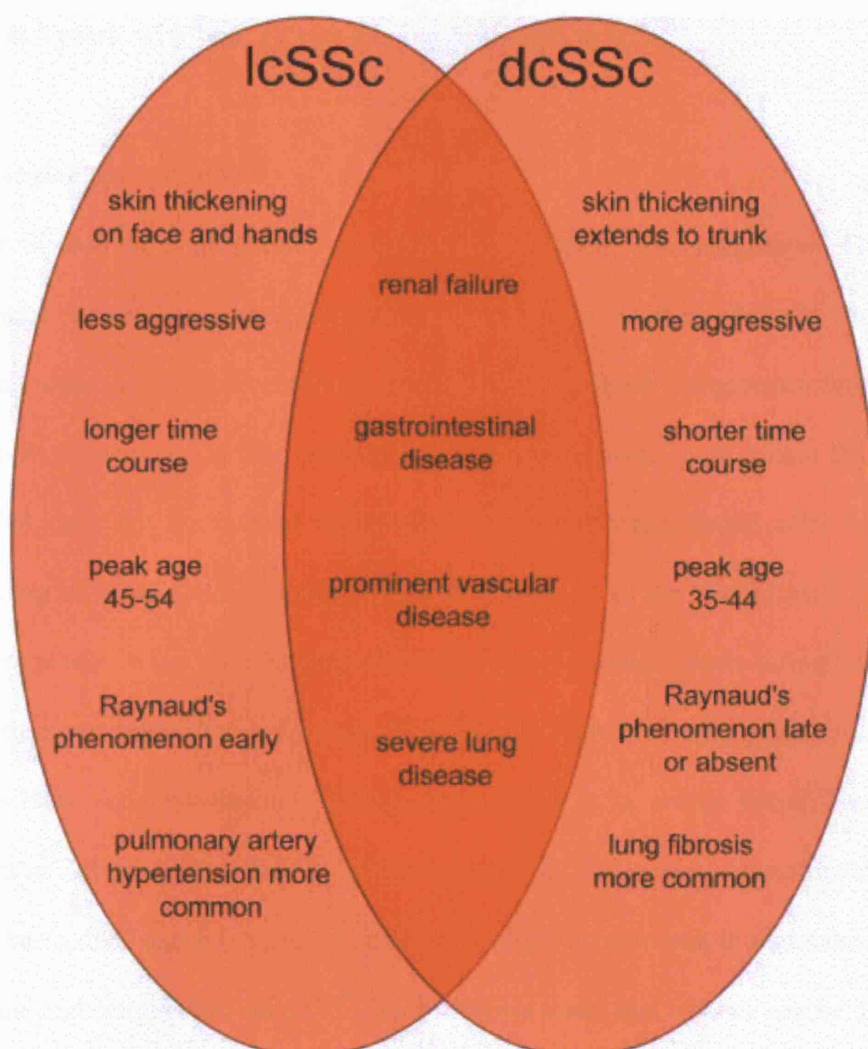
SSc is a worldwide condition. The female to male ratio has been reported as anywhere between 3 : 1 and 8 : 1, and the higher female preponderance is evident in all populations and in all age groups¹⁸⁻²¹. In the United Kingdom disease incidence is around 4 per 10⁶ per year, with a prevalence of 31 per 10⁶ per year^{20;22}. The precise aetiology of the condition is not known although environmental agents (such as organic solvents and polyvinyl chloride)^{23;24}, infectious agents (parvovirus B19 and cytomegalovirus)²⁵⁻²⁷, and genetic factors²⁸ have all been implicated.

1.1d. Clinical course

Both major SSc subgroups are progressive disorders characterised by excessive fibrosis of the skin, internal organs and vascular tree²². In lcSSc, the presentation typically starts with isolated Raynaud's phenomenon and this may be the only manifestation of disease for many years. Skin thickening usually heralds the onset of frank SSc, and classically occurs around the face and hands²². The disease may progress steadily over 10 years or more, with gradual progression of vascular disease and development of internal organ complications^{19;29}. Digital gangrene, necessitating amputation is relatively common and is a source of much morbidity³⁰⁻³². Oesophageal dysmotility leading to problematic reflux disease may also occur^{33;34}. Lung disease, either in the form of pulmonary artery hypertension or lung fibrosis, can be a particularly devastating complication³⁵⁻³⁸. Renal failure may also be a feature, often necessitating renal dialysis³⁹ (Figure 1.2).

The dcSSc subgroup shares many of the clinical features of lcSSc, but it is recognised as the more aggressive form. It is characterised by severe and progressive skin

Figure 1.2 The clinical features of lcSSc and dcSSc.



thickening which may extend to the trunk. Raynaud's phenomenon may occur but tends to be a later feature. After onset of symptoms, patients typically progress to advanced internal organ disease within 5 years; lungs, kidneys, and the gastro-intestinal tract may all be affected^{35;37;39}. Systemic sclerosis is associated with a significant reduction in

survival compared to the population mean, but although the dcSSc subgroup is thought to have a poorer prognosis than lcSSc^{18;19}, there appears to be no significant difference in survival at 5 years, with both groups ranging from 70% to 95%⁴⁰.

1.1e. Vascular disease in SSc

Vascular disease is a prominent feature of both major SSc subgroups. The precise mechanisms which cause vascular injury have yet to be fully defined, but various interesting theories have recently emerged. The heightened vasoconstricting potential seen almost universally in SSc patients has led investigators to speculate that vascular endothelial cells are the critical injury site⁴¹⁻⁴⁴. Vascular endothelial cells form a thin layer resting on a basal elastic lamina in the tunica intima of the arterial wall. These cells play a major role in the maintenance of normal vascular homeostasis through the release of a multitude of vasoactive mediators. Chief among these are endothelium-derived nitric oxide (edNO) and endothelin-1 (ET-1). The former is a potent vasodilator and has important anti-thrombotic effects⁴⁵, while the latter is a powerful vasoconstrictor. In the normal state, edNO and ET-1 provide a balance which is important in maintaining normal vessel tone and coagulation status^{46;47}, but in disease states this balance can be disrupted.

It is likely that endothelial dysfunction in SSc disrupts the interaction between these (and other) mediators in such a way as to increase the arterial vasoconstricting potential. In addition, endothelial dysfunction disrupts the normal coagulation cascade such that these patients become pro-thrombotic at a microvascular level. This leads to repeated microvascular occlusions with associated ischaemia/reperfusion injury, resulting in a vicious cycle that frequently ends in digital ulceration and gangrene^{43;44;48-57}. In fact

endothelial injury is no longer thought to be confined to the digital microcirculation, and is now thought to play a much wider role in SSc. For example, endothelial dysfunction of the pulmonary circulation is thought to contribute to lung disease secondary to SSc^{58;59}.

Notwithstanding the critical role of endothelial dysfunction, it is unlikely to be the sole event in SSc vascular injury. Given that SSc is primarily a disease of widespread excessive fibrosis it is reasonable to argue that fibrotic vascular disease is as prevalent as endothelial injury. The tunica media of the arterial wall consists of a layer of smooth muscle cells embedded in a collagen rich extra-cellular matrix, and this layer is likely to be just as susceptible to the generalised fibrotic reaction as other organ systems^{60;61}. It is possible that some pro-fibrotic arterial injury occurs *secondary* to prolonged endothelial dysfunction, whereby repeated episodes of ischaemia/reperfusion driven inflammation causes excessive production of potent fibroblast chemo-attractants such as transforming growth factor- β (TGF- β), although there is currently no evidence to support occurrence of this phenomenon. However, with the recent emergence of evidence of genetic abnormalities in SSc (specifically of the gene that encodes fibrillin - an important component of the arterial extra-cellular matrix), *primary* pro-fibrotic vascular disease is likely to play an important role alongside endothelial injury⁶²⁻⁶⁷.

1.2 Aims of this thesis

Systemic sclerosis is a complex condition, and there is much ongoing research involving a wide range of fields including cellular and molecular biology, immunology, pathology and physiology. In order to acquire a fuller understanding of these issues we commenced our study with a literature review of vascular function in SSc (Chapter Two).

1.2a. First Hypothesis – Endothelial Dysfunction

Upon completion of this review, it became apparent that while numerous investigators have conducted *in-vitro* analyses of endothelial function, as yet no group had convincingly demonstrated endothelial dysfunction *in-vivo* in SSc patients. It also became apparent that although SSc subgroups can often vary markedly in their clinical profile and prognosis, very few investigators had sought to identify any difference in endothelial function between lcSSc and dcSSc. Our *first hypothesis* was twofold: first, that endothelial dysfunction can be demonstrated *in-vivo* in a sample of SSc patients; second, that the dcSSc subgroup displays a greater degree of endothelial dysfunction in-line with it's more aggressive clinical profile.

To prove this hypothesis, an applicable method was needed that allowed accurate assessment of *in-vivo* endothelial function. This proved challenging because while there are numerous methods currently available, they differ widely in terms of protocol and region of study, and none were currently in use in our department. Furthermore, there is no universally established convention to assist in the decision as to which method would be applicable to a sample of SSc patients. This problem was approached by conducting a second critical review of the literature. The aim for this review was to identify all the currently used methods for detecting endothelial function *in-vivo*, and to study how they had been applied for the investigation of vascular disease in general. This forms Chapter Three of this thesis.

After conducting this review, two non-invasive methods were chosen to apply to samples of SSc patients. These were brachial artery flow-mediated dilatation (FMD) and

laser Doppler iontophoresis (LDI), and they are described fully in Chapters Four, Five and Six.

1.2b. Second Hypothesis – Altered Arterial Biomechanics

While conducting the literature review of vascular function in SSc (Chapter Two), the potential significance of *fibrotic* vascular disease as a separate and co-existent entity from endothelial dysfunction became increasingly apparent. In theory, fibrotic vascular disease should be reflected as an impairment of arterial biomechanics; and in fact in a previous study Cheng *et al.* have demonstrated altered elasticity parameters of the common carotid artery in SSc patients⁶⁸. Our *second hypothesis* was twofold: first, that if there is indeed a genetic (or other acquired) abnormality causing pro-fibrotic vascular disease in SSc then this should be a system-wide phenomenon, and thus apparent as altered arterial elasticity in other vessels in addition to the common carotid artery; and second, that pro-fibrotic vascular disease should be manifest as altered arterial *viscous* as well as elastic properties.

To attempt to prove this hypothesis, several well established algorithms that allow determination of arterial elastic properties, namely diametric compliance, Peterson's elastic modulus and stiffness index, were applied to SSc subgroups and healthy controls. These are described in Chapters Four and Seven. Arterial *viscoelastic* properties were determined using pressure-flow (hysteresis) loops, and this is described in Chapters Four and Eight.

Chapter Two

A Review of the Mechanisms of Vascular Injury in SSc

2.1 Introduction

Striking vascular abnormalities are a hallmark feature of all types of systemic sclerosis. Individuals with the lcSSc subset typically manifest Raynaud's phenomenon many years before onset of frank scleroderma symptoms. Repeated and prolonged peripheral vasospasm frequently leads to painful digital ischaemia, ulceration and gangrene, that often requires surgery and amputation⁶⁹. But vascular disease is known to extend beyond the peripheral circulation, and while it's role in overall disease evolution is yet to be fully defined, it is likely that some internal organ complications of SSc may be the result of end-organ vascular injury⁷⁰. Indeed, scleroderma renal crisis, often an early feature of dcSSc and frequently a cause of mortality in this group, is thought to have an underlying vascular pathophysiology⁷⁰⁻⁷². And there is evidence that dysregulated vasomotor tone in the pulmonary circulation causes pulmonary artery hypertension secondary to lcSSc^{59;70;73}.

The nature of SSc vascular dysfunction was long thought to be essentially a heightened vasoconstricting potential, particularly affecting the microcirculation. This belief was largely based on observations of patients with Raynaud's phenomenon secondary to SSc, and also on empirical data from patients treated with vasodilating agents^{69;74;75}. It is now increasingly clear that vascular pathophysiology in SSc involves several mechanisms, which together have the potential to affect *all* parts of the systemic vascular tree. First, there is evidence of abnormalities of multiple components of the vessel wall including endothelial cells^{41;76-78}, smooth muscle cells⁴⁹, and extra-cellular matrix (ECM)^{68;79} (Figure 2.1). Second, these changes are not likely to be confined to the microcirculation as previously thought, but are probably a feature of vessels of various

sizes, including medium-sized muscular arteries⁴⁹ and large elastic arteries^{68;79}. Third, significant changes occur within the vessel lumen, probably secondary to endothelial dysfunction, and resulting in a heightened tendency to coagulate^{80;81}. All this equates to a disease state in which there is abnormal vasoconstriction, reduced arterial elasticity, and prolonged and repetitive microvascular occlusions. This leads to significant haemodynamic disturbances and ischaemic injury, both of which are major factors in disease progression.

Figure 2.1. Diagram summarising the effect of SSc on a typical artery.

Tunica Intima

Consists primarily of a layer of endothelial cells. Endothelial dysfunction impairs release of vasoactive mediators such NO and ET-1. This increases the vasoconstricting potential and causes clotting abnormalities.

Tunica Media

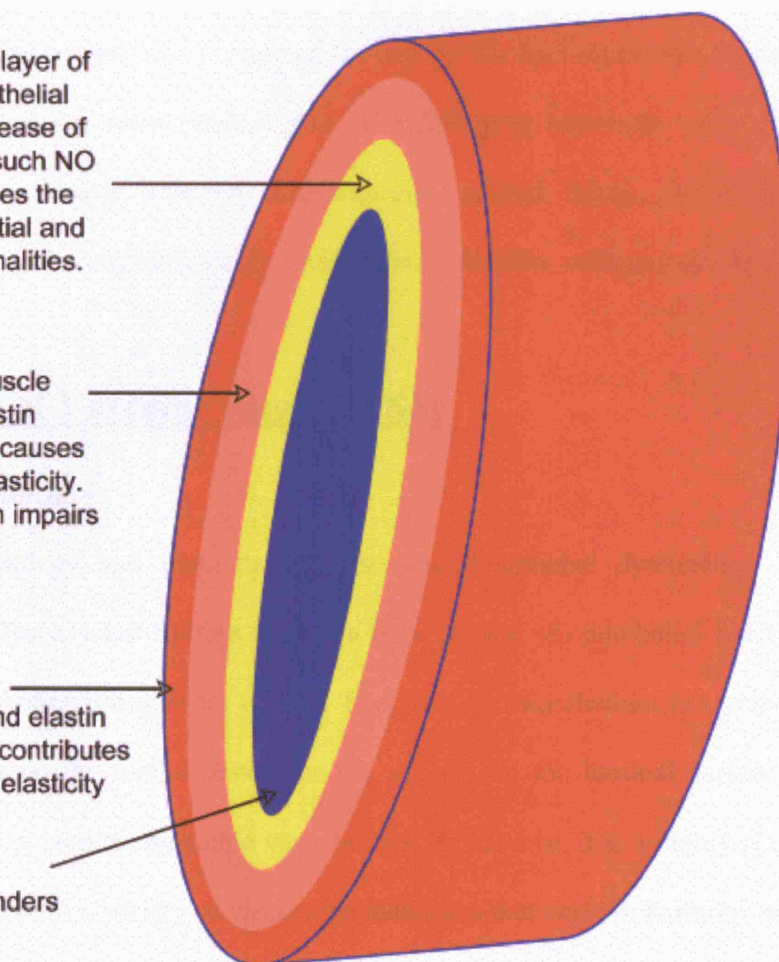
Consists of smooth muscle cells, collagen and elastin fibres. Marked fibrosis causes alterations in vessel elasticity. Endothelial dysfunction impairs regulation of smooth muscle tone.

Tunica Adventitia

Consists of collagen and elastin fibres. Marked fibrosis contributes to alterations in vessel elasticity

Lumen

Platelet dysfunction renders blood hypercoagulable



This review seeks to shed light on the mechanisms of vascular injury implicated in SSc. These mechanisms are complex and highly intertwined, and the evidence is occasionally conflicting. But they can be broadly divided into those that involve injury to vascular endothelial cells and those that promote fibrotic vascular disease, and these are outlined in the section below (and summarised in Table 2.1). In subsequent sections there is a more in-depth analysis of how mediators of normal vascular function become dysregulated and contribute to SSc vascular disease.

2.2 Search Methodology

Literature search for this review was conducted on-line by the lead researcher (myself) using Medline, accessed via www.PubMed.gov. The following keywords were used: scleroderma, systemic sclerosis, vascular, microvascular, arterial, injury, endothelium, endothelial dysfunction, fibrosis, nitric oxide, endothelin-1, fibrillin, collagen and elastin.

2.3 Mechanisms of Vascular Injury in SSc

2.3a. Endothelial dysfunction

Endothelial cell physiology and methods for assessing endothelial dysfunction are discussed in Chapter Three, what follows here is a brief outline of endothelial function and how endothelial abnormalities relate to SSc. The vascular endothelium is a cellular monolayer that makes up the *tunica intima*, and is present on the luminal surface of vessels of all sizes throughout the systemic vascular tree. In essence, it is a highly-active paracrine organ that secretes a variety of vasoactive mediators that work in harmony with each other, and with the sympathetic nervous system, to maintain normal vasomotor tone

and coagulation status⁴⁵. Endothelial *dys*function is known to be a precursor to most cardiovascular diseases, including coronary artery disease and atherosclerotic peripheral vascular disease.

Although yet to be conclusively demonstrated *in-vivo*, endothelial dysfunction is highly likely to be a crucial component in the vascular pathogenesis of SSc^{41;52;76-78;82-84}. The triggering event for endothelial injury is not known, although several theories have emerged. It has been postulated that a class of autoantibody, distinct from ANA, Scl-70 and other rheumatic auto-antibodies, may specifically target vascular endothelial cells in autoimmune vascular disorders; these have been dubbed anti-endothelial cell auto-antibodies (AECA)^{54;56;85}. Carvalho *et al.*, in a study where they treated cultured human endothelial cells with AECA positive sera drawn from SSc patients, have demonstrated that AECA causes endothelial cell activation and release of inflammatory mediators, in particular IL-1, with subsequent lymphocyte infiltration⁸⁵. Another putative effect of AECA may be to induce spontaneous endothelial cell apoptosis, by binding to cell surface receptors. Worda *et al.* have demonstrated, in an avian model of SSc, that endothelial cells undergo apoptosis in the presence of AECA⁵⁶.

It is likely that AECA are damaging to endothelial cells but it is not yet clear whether they cause primary endothelial injury, or secondary injury in response to other immunologic stimuli, such as a latent effect following a previous viral infection. Certainly, there is evidence to support a viral aetiology for SSc, possibly involving latent cytomegalovirus (CMV) infection of endothelial cells⁸⁶⁻⁹⁰. Lunardi *et al.* have shown that antibodies with CMV affinity have the ability to induce apoptosis in endothelial cells cultured from SSc subjects⁹¹. No studies have yet been conducted to try and identify any

homology between AECA and these “anti-CMV” antibodies. A possible scenario is that CMV infection, together with an environmental or genetic factor (possibly a mutation of the fibrillin-1 gene), triggers the initial immune response, with AECA produced as a by-product. In this scenario AECA functions as a potent endothelial activator, and the inflammatory stimulus is propelled forward towards endothelial injury.

Whatever the precise aetiology of endothelial injury, once established it sets in motion a vicious cycle that is critical to the evolution of SSc vascular disease. Dysregulated release of vasoactive mediators leads to the heightened vasoconstricting potential seen universally in these patients. There is activation of leukocytes in end-organs and in the vessel walls themselves, with the release of pro-inflammatory cytokines and growth factors^{54;89;92}. There is a surge in fibroblast activity, causing excessive collagen synthesis and extra-cellular matrix (ECM) deposition^{78;93-95}. Furthermore, endothelial injury dysregulates platelet function and causes abnormal release of clotting factors, leading to a hyper-coagulable state and repeated microvascular occlusions⁸¹. The resulting ischaemia/reperfusion episodes generate reactive oxygen species, exacerbating endothelial cell injury⁴². As the disease progresses, the repeated inflammatory stimuli cause arterial smooth muscle proliferation and inter-cellular scarring, resulting in an abnormal thickening of the *tunica media*⁷⁶. This increases arterial stiffness, adds to the vasoconstricting potential, and further contributes to endothelial injury.

2.3b. Fibrotic Vascular Disease

The *tunica media* is composed of a circumferential arrangement of smooth muscle cells embedded in a collagen and proteoglycan rich extra-cellular matrix (ECM). This layer,

assisted by the thin outermost *tunica adventitia*, is responsible for sustaining a resilient arterial structure. The processes that trigger abnormal stiffening usually fall into broad and identifiable categories such as mechanical stress, inflammation, genetic factors, nicotine toxicity and hormonal influences. However, ultimately any factor that affects arterial stiffness must impact directly on the relationship between arterial ECM and medial smooth muscle cells⁹⁶.

Under normal circumstances, the collagenous ECM of the tunica media maintains smooth muscle cell alignment and integrity, while providing a suitable framework in which smooth muscle cells can function normally – an essential requirement for the maintenance of normal vessel tone⁹⁷. Smooth muscle cells are linked to elastic lamellae of the ECM by microfibrils composed of *fibrillin*, and also by various collagen types including I, III, IV and VI⁹⁸. Collagen, in particular types I and III, has a high tensile strength and elastic modulus, but has a low strain. Other molecules that have important roles in this interaction include elastin, glycoprotein and proteoglycans⁹⁸. The ECM provides a scaffold in which individual smooth muscle cells can interact in such a way that maintains a normal vasomotor tone, an essential requirement for pulse wave propagation in a healthy arterial tree.

Any disease process that interferes with this interaction, for example by means of excessive collagen deposition or by affecting linking molecules such as fibrillin, will result in abnormalities of arterial stiffness. These effects can be demonstrated in arteries of various sizes including large vessels such as the aorta⁹⁹⁻¹⁰¹ and medium-sized muscular arteries such as the coronary artery¹⁰². Indeed, increased arterial stiffness is a well recognised feature of cardiovascular disease in general, and it is known to occur

secondary to essential hypertension, advancing age, presence of atherosclerosis, and menopause^{61;99;100}. Increased stiffness is also classically found in genetic connective tissue disorders such as Marfan's syndrome, which is associated with aortic dilatation⁶⁰. Systemic sclerosis is primarily a disease of excessive fibrosis and uncontrolled collagen deposition, caused by biosynthetically activated fibroblasts. These changes are known to occur in the skin and internal organs of patients⁶⁹, but are also likely to occur in arterial walls. Preliminary evidence for this is provided by Cheng *et al.*, who have demonstrated reduced compliance and increased stiffness in the carotid arteries of SSc patients^{68;79}. But given the structural importance of collagenous ECM in arteries of various sizes, it is probable that the arterial elasticity changes observed in SSc are not confined to large vessels such as the common carotid, but are in fact present throughout the vascular tree including medium-sized muscular arteries such as the brachial artery.

Table 2.1. The mechanisms of vascular injury in systemic sclerosis.

Injury Mechanism	Site	Aetiology	Effect
1. Endothelial dysfunction	Endothelial cells of the tunica intima.	?AECA	Dysregulated vasomotor tone.
	Principally affecting the microcirculation.	?Viral infection	Heightened constricting potential.
	Possibly affecting medium-sized arteries (eg. brachial).		Altered clotting status. Repeated microvascular occlusions.
2. Fibrotic vascular disease	ECM and smooth muscle cells of the tunica media and tunica adventitia.	?Fibrillin gene defects	Dysregulated ECM and SMC interactions.
	Known to affect large elastic arteries such as the carotid.	?Secondary to endothelial cell dysfunction.	Increased arterial stiffness.
	May affect medium-sized arteries (eg. brachial).		

2.4 Critical Mediators of Vascular Injury in SSc

2.4a. The Role of Nitric Oxide in SSc

The release and mechanism of action of nitric oxide, in the context of endothelial function assessment, will be discussed in Chapter Three; what follows here is an outline of how dysregulated release of nitric oxide relates to SSc. Nitric oxide is a potent endothelium-derived vasodilator, and the balance that it strikes with various endothelium-derived vasoconstrictors, and the sympathetic nervous system, is critical in the maintenance of normal vasomotor tone⁴⁵. It is manufactured via nitric oxide synthase (NOS) from the precursor L-arginine¹⁰³. In mammals, there are at least 3 recognised isoforms of NOS: endothelial, inducible and neuronal^{45;104}. The neuronal isoform is not known to play an important role in SSc pathogenesis and will not be considered further here. The endothelial isoform (eNOS) is expressed constitutively by endothelial cells, under regulation by the vasoconstrictor endothelin, neurotransmitters such as acetylcholine and bradykinin, and by adjacent intra-luminal physical forces (*shear stress*)⁴⁵. The inducible isoform (iNOS) is expressed by endothelial cells, as well as smooth muscle cells, macrophages, fibroblasts and other inflammatory cells. Production of iNOS is regulated by inflammatory mediators and cytokines^{105;106}.

In healthy individuals, endothelium-derived nitric oxide (henceforth referred to as edNO) produced via the eNOS isoform has a highly protective role. It is a vasodilator, but it also has important anti-thrombotic effects¹⁰⁷. It suppresses platelet aggregation, reduces cellular adhesiveness, and has anti-migratory and anti-proliferatory influences on leukocytes and smooth muscle cells¹⁰⁷. Production of edNO is utterly dependent on the presence of an intact endothelium⁴⁵, and dysregulated release of edNO leads to abnormal

vasoconstriction, vascular inflammation, smooth muscle proliferation, and a heightened coagulating tendency^{44;45;76;78;81;108}. The resulting inflammation and ischaemia/reperfusion injury causes the formation of reactive oxygen species⁴². Under normal circumstances, a steady basal release of edNO is thought to have a cytoprotective effect against free-radical induced injury^{109;110}, but this is lost when endothelium is dysfunctional. These phenomena are well recognised in atherosclerotic disease¹¹¹⁻¹¹⁴, or after prolonged exposure to the major cardiovascular risk factors (smoking¹¹⁵⁻¹¹⁷, hyperlipidaemia¹¹⁸⁻¹²⁰, hypertension^{58;121;122}, diabetes mellitus^{123;124} and obesity^{122;125}).

The role played by nitric oxide in SSc is complex, and apparently contradictory. Given the digital vasospasm and peripheral ischaemia observed in SSc, it is logical to assume that there is reduced production and/or bioavailability of nitric oxide⁹². In fact there is evidence that nitric oxide levels in SSc are *raised*^{48;53;55;57} as well as reduced^{44;126}, and this has led to interesting speculation about the varying roles of different types of nitric oxide. Several investigators have reported reduced serum levels of edNO metabolites^{44;126}. The most compelling evidence comes from Kahaleh *et al.* who reported reduced levels of eNOS mRNA in endothelial cells derived from SSc patients⁴⁴.

Paradoxically, numerous studies have demonstrated that levels of total nitric oxide are actually *raised* in SSc^{44;48;53;55;57}, and that disease activity, as measured by serum markers of endothelial dysfunction, is directly related to total nitric oxide level⁵⁷. This is likely a result of increased expression of the inducible isoform of NOS¹²⁷. Inducible NOS is expressed in a variety of cells, particularly those associated with inflammation; thus raised levels can be detected in, among others, endothelial cells, skin fibroblasts, and alveolar macrophages^{128;129}. Nitric oxide appears to play a dual role in SSc, with the

eNOS type having a broadly protective role and the iNOS type possibly having an injurious effect.

The precise mechanism by which inducible nitric oxide inflicts cellular injury is not yet clear, but there is evidence of excessive production of reactive oxygen species. As the inflammatory process proceeds, free-radicals react with nitric oxide produced via iNOS to generate *peroxynitrite*, a molecule that causes particularly wide-ranging tissue damage⁴². Furthermore, as SSc progresses and inflammation becomes more marked, production of nitric oxide in endothelial cells appears to gradually shift from the eNOS moiety to iNOS¹²⁸. Thus the ratio of “protective” nitric oxide to “damaging” nitric oxide is decreased. The role of nitric oxide and the other critical mediators in SSc endothelial dysfunction are summarised in Table 2.2.

Table 2.2 Critical vasoactive mediators in SSc endothelial dysfunction

	Nitric oxide	Endothelin-1 (ET-1)	Prostacyclin (PGI₂)
Normal effect	↓ Vasomotor tone ↓ Platelet aggregation ↓ Leukocyte migration ↓ SMC proliferation	↑ Vasomotor tone ↑ Inflammatory mediators ↑ Fibroblast activity ↑↓ Neurovascular activity	Arachidonic acid metabolite ↓ Vasomotor tone ↓ Thrombotic potential
Role in SSc	Loss of vasodilation ↓ Endothelial isoform ↑ Inducible isoform ↑ Reactive O ₂ species	Vasoconstrictor ↑ Fibroblast activity ↑ ET-1 expression ↑ ET _A : ET _B receptor ratio	Therapeutic importance

2.4b. Endothelin: Vasoconstrictor and Fibrosis Promoter?

Closely resembling the sarafotoxins that constitute snake venom⁴⁷, endothelin is the main vasoconstricting agent produced by endothelial cells. The endothelin peptide exists in three isoforms (ET-1, ET-2 and ET-3)⁴⁶, and the significant isoform in humans is the 21 amino acid peptide ET-1¹³⁰. This peptide has critical homeostatic roles in cardiovascular, renal and pulmonary physiology, and is also important in promoting normal growth and development^{131;132}.

Although predominantly expressed by endothelial cells, ET-1 is also expressed by inflammatory cells such as fibroblasts, macrophages, mast cells and polymorphonuclear leukocytes^{133;134}. It is manufactured in a process involving calcium-dependent protein kinase C^{135;136}, under the regulation of a number of chemical and physical forces. Stimulants to expression include hypoxia, low shear stress, and angiotensin II (Ang II). Inhibitors include edNO, prostacyclin, atrial natriuretic peptide and low blood flow^{135;137;138}. The original precursor molecule to ET-1 is pre-proendothelin, from which is derived the 38 amino acid peptide proendothelin (or “big ET-1”). ET-1 itself is cleaved from proendothelin by endothelin-converting enzyme^{135;139;140}. ET-1 binds to one of two types of cell surface receptor, each belonging to a superfamily of 7-transmembrane G-protein coupled receptors, and designated ET_A and ET_B^{141;142}.

ET-1 exerts considerable influence on vascular function in a variety of ways (Table 2.2). Upon release by endothelial cells it acts in a paracrine manner, binding directly to ET_A receptors on vascular smooth muscle cells, stimulating contraction^{133;143}. As the main endothelium-derived vasoconstricting agent, it provides an important balance with edNO in maintaining normal vasomotor tone. It is also a powerful potentiator of

neurotransmitters such as acetylcholine and noradrenaline^{144;145}, a feature which gives ET-1 considerable influence over neural control of vasomotor tone¹⁴⁴⁻¹⁵⁰. ET-1 also stimulates the inflammatory mediator phospholipase A₂, thus exerting important effects on endothelial cell arachidonic acid metabolism. In this way, ET-1 appears to counteract its direct vasoconstricting effect on vascular smooth muscle, by indirectly promoting arterial vasodilation and inhibiting platelet aggregation (via the inflammatory mediator prostacyclin)^{151;152}. Last but not least, ET-1 has a strong association with pro-fibrotic activity. It stimulates endothelial cells to produce fibronectin, a potent fibroblast chemotactic agent, and potentiates fibroblast collagen synthesis^{43;94;153}.

In SSc, it is postulated that ET-1 is partly responsible for the heightened vasoconstricting potential^{43;133;154}. Indeed, numerous investigators have demonstrated higher plasma levels of ET-1 in SSc patients versus healthy controls^{51;155-159}. Higher levels have also been reported as a particular feature of the dcSSc subtype^{51;157}, and also in those with PAH secondary to lcSSc¹⁵⁶. Interestingly, abnormalities in the expression of ET-1 and its receptor are implicated in generalised (non-vascular) SSc pro-fibrotic activity. In an *in-vitro* study, Shi-Wen *et al.* demonstrated down-regulated expression of ET_A receptors in fibroblasts cultured from SSc lesional skin biopsies; they suggest that the relative ET_A: ET_B ratio may contribute to the abnormal, pro-fibrotic phenotype that these cells display^{43;93}. This does suggest a dual role for ET-1 (as a potent vasoconstrictor *and* an important fibrosis promoter), but the *in-vivo* significance of this pro-fibrotic activity is not yet clear, particularly in relation to other mechanisms such as fibrillin gene defects.

Improved understanding of the role of ET-1 and its receptors is set to have profound therapeutic implications for SSc patients. Preliminary results from the RAPIDS-1 study (Randomised Placebo Controlled Study on the Prevention of Digital Ulcers secondary to SSc), demonstrate that a 16 week course of the oral dual specificity ET_A and ET_B receptor antagonist *bosentan* can inhibit the vasoconstricting effect, and actually prevent onset of new digital ulcer formation, particularly in high risk patients with pre-existing ulceration¹⁶⁰. Similar encouraging results have been reported for the treatment of PAH secondary to SSc¹⁶¹.

2.4c. Prostacyclin: An Important Therapeutic Role

Prostacyclin (PGI₂) is the main product of endothelial cell arachidonic acid metabolism, and it has potent vasodilating and anti-thrombotic activities. It is a member of the prostaglandin family of lipid mediators, thus PGI₂ is a 20-carbon unsaturated carboxylic acid with a cyclopentane ring¹⁶². Its main role in vascular function appears to be to maintain normal vascular homeostasis by opposing vasoconstricting and pro-thrombotic mediators such as ET-1 and the arachidonic acid metabolite thromboxane A₂¹⁶²⁻¹⁶⁴.

Reduced expression of PGI₂ has been implicated in peripheral vascular disease secondary to atherosclerosis¹⁶⁵. But there is as yet no evidence to suggest a direct role for PGI₂ in vascular disease secondary to SSc, although it may have a supporting role secondary to ET-1 dysregulation^{151;152}. The significance of PGI₂ is in the therapeutic value of its analogues (Table 2.2). The intravenous prostacyclin analogue *iloprost* is widely and successfully used in patients with SSc vascular disease^{36;74;75;166}. It is routinely administered to those with severe digital vasospasm refractory to treatment with oral

calcium-channel blockers^{50;75}. It is also useful for treating patients with severe digital ulceration and gangrene, as a last ditch effort to avoid surgery^{50;74;166}. One area where prostacyclin analogues have proved particularly useful is in the treatment of advanced PAH secondary to SSc, where it is given as a continuous intravenous infusion³⁶.

2.4d. The Role of Cytokines : Transforming growth factor - β

Transforming growth factor- β (TGF- β) is a potent fibroblast chemo-attractant that is released by fibroblasts, endothelial cells and other inflammatory cells as part of a cellular response to injury¹⁶⁷. In essence, it mediates wound healing and scar formation by powerfully promoting the synthesis of collagen. TGF- β has attracted much recent interest as a potential mediator of fibrotic activity in SSc.

First described in 1978 in a murine sarcoma model¹⁶⁸, it is known to exist in 3 isoforms in mammals – TGF- β 1, TGF- β 2 and TGF- β 3¹⁶⁹. They are homodimeric molecules synthesised from larger precursor proteins, and may be released in either a latent or an active form¹⁷⁰. The signalling pathway that each isoform follows determines it's function. This involves first binding to one of two high affinity TGF receptors (T β R-I and T β R-II) and then activating a group of downstream signalling proteins, known collectively as *Smads*¹⁷¹. Smads are activated by phosphorylation and are translocated to the nucleus where they can influence gene expression based on the initiating TGF- β ligand¹⁷¹.

Most investigators agree on the importance of TGF- β as a pro-fibrotic stimulant in SSc, however, the reports are somewhat conflicting. At least one study has identified elevated circulating levels of TGF- β ¹⁷², and there are reports of TGF- β immunoreactivity

in lesional skin biopsy samples¹⁷³⁻¹⁷⁵. Experiments using mononuclear cells cultured from bronchoalveolar lavage leukocytes have shown increased expression of TGF- β ¹⁷⁶, but these findings have been contradicted by other groups¹⁷⁷. Several studies have utilised *in-situ* hybridisation techniques to successfully identify increased TGF- β mRNA expression in perivascular tissue and dermal tissue¹⁷⁸, but once again these findings have not been universal^{179;180}. The reason for the failure to consistently identify TGF- β in SSc patients may be partly explained by differences in methodology, but are probably also a reflection of the variable stage of the disease at time of study. Nevertheless, the importance of TGF- β is underlined by recent studies that look more closely at the TGF- β signalling pathway. In an elegant experiment using dcSSc dermal fibroblasts, Ihn *et al.* demonstrated over-expression of high affinity TGF- β receptors, and then demonstrated inhibition of this over-expression by anti-TGF- β antibodies; thus providing direct evidence of the significance of TGF- β in maintaining pro-fibrotic activity¹⁸¹.

In SSc vascular disease, the precise relationship between endothelial dysfunction and TGF- β is not known. It is possible that endothelial dysfunction sets in train a series of events that triggers overproduction of TGF- β , and so medial and adventitial fibroblast stimulation may be a secondary event. Probably more likely is that endothelial dysfunction and fibroblast stimulation are separate events, with vascular pro-fibrotic activity occurring contemporaneously with skin and internal organ pro-fibrotic activity. Once TGF- β is released, local fibroblasts are activated and stimulate release of more TGF- β . Thus an *autocrine loop* is established that results in continuing fibrosis^{66;182}.

2.4e. The role of cytokines : Connective tissue growth factor

Connective tissue growth factor (CTGF) is a cysteine-rich matricellular protein produced by fibroblasts in response to stimulation by TGF- β , and is another pro-fibrotic cytokine that has been implicated in SSc pathogenesis^{171;183-185}. It is thought that while TGF- β provides the initial, powerful pro-fibrotic stimulus, CTGF is responsible for ongoing and chronic fibrosis. There is evidence of TGF- β in pre-lesional SSc dermal biopsies, but less convincing evidence from established and chronic lesions. This suggests that ongoing fibrosis is mediated by a secondary cytokine, postulated to be CTGF^{171;183-185}.

Increased expression of CTGF has been demonstrated in serum¹⁸⁶, lesional skin biopsies¹⁸⁷ and bronchoalveolar lavage cells¹⁸⁸ of SSc patients. Interestingly, it has been found to be released by endothelial cells, prompting speculation that it is an important link in the chain of molecular events that occur between endothelial cell activation and increased fibroblast activity¹⁸⁵.

2.4f. Mutations of the Fibrillin Gene

Since the ECM plays a critical role in maintaining arterial biomechanical properties, it follows that mutations of ECM component proteins will have detrimental effects on arterial function. Elastin and microfibrillar proteins are essential ECM components that can be found in all tissues that need to withstand strain, such as medium/large arteries. Elastic fibres are composed of an insoluble amorphous core of elastin surrounded by a lattice of microfibrils¹⁸⁹. Microscopy studies of aortic tissue provide visual evidence that elastic fibers form thick concentric lamellae in the tunica media with interlaminar connecting fibers scattered radially through the vessel wall, while microfibrils form a

complex meshwork through medial and adventitial layers⁶⁰. It is thought that this arrangement facilitates the role of microfibrils as flexible links that make the vessel wall work as a unit, while elastic fibers allow smooth dilatation and recoil⁶⁰.

The major microfibrillar components are fibrillin 1 and fibrillin 2^{190;191}. They are both nearly identical in structure, but have somewhat different temporal and spatial expression patterns. Fibrillins are mainly composed of tandem repeats of calcium-binding epidermal growth factor-like (cbEGF) domains interspersed by cysteine-rich sequences with homology to a motif found in the TGF- β binding proteins (TB motif) or hybrids between cbEGF and TB motifs (Fib motif). Head to tail polymerisation of individual fibrillin molecules gives rise to a “bead-on-a-string” appearance of microfibrils^{60;190-192}.

The idea that fibrotic activity in SSc may be explained by mutations of the gene that encodes fibrillin-1 (*fbn1*) has generated much recent interest. The extensively studied murine model of SSc (tight skin mice, Tsk), demonstrates a typical phenotype of skin and internal organ fibrosis^{63;64}. The Tsk phenotype is the result of mutations of the murine *fbn1* gene, indicating that defects of this gene can induce excessive fibroblast activity. In humans, mutations of the *fbn1* gene have long been recognised as the underlying cause of the autosomal-dominant connective tissue disorder Marfan’s syndrome⁶⁰. Evidence of *fbn1* gene mutations in SSc first came from studies looking at the Choctaw Native Americans. This population has an unusually high prevalence of SSc, significantly higher than other racial groups in similar geographical locations, and over the years has provided a useful group in which to investigate potential genetic aetiological factors for the disease^{62;65}. Using microsatellite markers in and around the *fbn1* gene on chromosome 15q, Tan *et al.* were able to define a 2-cM haplotype that was strongly associated with

SSc⁶⁵. In a subsequent study, they found similar single nucleotide polymorphisms (SNP) in the 5'-untranslated region of *fbn1* in both Choctaw and Japanese SSc patients⁶⁷.

In fact, the Marfan's analogy presents an interesting paradox. In this condition, the *fbn1* gene mutation results in a relative paucity of ECM microfibrils⁶⁰. By contrast in SSc, despite evidence of *fbn1* gene defects, there is evidence that microfibrils are abundant but possibly unstable¹⁸². Evidence from Tsk suggests that microfibrils that incorporate the mutant fibrillin protein are more prone to proteolytic degradation¹⁹³. If a similar process were to occur in humans, then this would be one possible explanation of SSc fibrotic activity. It is postulated that proteolytic degradation of the mutant fibrillin microfibrils by matrix metalloproteinases and serine proteases, leads to inappropriate release of TGF- β , activating fibroblasts and kickstarting the *autocrine loop*^{182;194}.

2.5 Summary

Since we presently await the emergence of a definitive triggering agent for SSc, this review is essentially an analysis of the downstream cellular mechanisms that ultimately disrupt normal arterial function and lead to a typical SSc morphology (digital ischaemia, gangrene and internal organ failure). The mechanisms that are implicated in SSc vascular injury are highly complex, and involve several closely-related but distinct components of arterial function. Given our current level of knowledge of normal arterial function, making sense of these wide-ranging mechanisms and the way in which they interact, is quite a challenge. One convenient way in which these processes can be categorised is into those that involve primary injury to vascular endothelium, and those that involve primary fibrotic vascular injury.

It is highly likely that endothelial dysfunction is a critical step in SSc vascular pathogenesis, although this has yet to be conclusively demonstrated *in-vivo*. The triggering event is unknown (although latent CMV virus infection and AECA are thought to play a role); but the downstream effects, caused by dysregulated release of edNO and ET-1, are unmistakable. There results a heightened vasoconstricting potential, and a hyper-coagulable state that causes repeated microvascular occlusions, both of which are typical features of SSc.

Since SSc is essentially a disease of widespread excessive fibrotic activity, it is likely that this fibrosing process also contributes to SSc vascular disease. Indeed, a previous study group from our own department have already demonstrated increased carotid arterial stiffness *in-vivo* in SSc⁶⁸. The underlying cause is thought to be one or more mutations of the gene that encodes fibrillin-1, an essential arterial ECM component. This leads to microfibrillar instability and degradation, with inappropriate release of TGF- β and continued fibroblast activation. The result is uncontrolled collagen synthesis and eventual disruption of normal smooth muscle cell/ECM interactions, which is manifest as increased arterial stiffness.

An interesting issue is the relationship between primary fibrotic vascular disease and fibrotic vascular disease secondary to endothelial dysfunction. Dysregulated release of edNO and ET-1 by endothelial cells results in medial fibroblast activation and smooth muscle cell proliferation. Studies into atherosclerotic vascular disease provide evidence that prolonged endothelial dysfunction leads to structural changes within the arterial wall that eventually manifests itself as increased arterial stiffness. As yet it is not clear to what extent fibrosis secondary to endothelial dysfunction contributes to SSc vascular disease.

Chapter Three

Endothelial Function Testing *In Vivo* :

A Review of Available Methods

3.1 Introduction

An intact vascular endothelium is essential in maintaining short term control of arterial tone and coagulation status, and longer term control of smooth muscle cell proliferation and extra-cellular matrix production. Injury to the vascular endothelium is likely to be a preliminary event in most if not all vascular disease. Endothelial dysfunction has been implicated in diabetes mellitus, essential hypertension, hypercholesterolaemia, systemic sclerosis and primary Raynaud's phenomenon. Furthermore, it is postulated that endothelial dysfunction is a precursor to frank atherosclerosis¹⁹⁵⁻¹⁹⁸; indeed it has been identified *in-vivo* in healthy individuals exposed to cardiovascular risk factors such as smoking, obesity and age, and appears to be less prevalent in pre-menopausal women.

Cardiovascular disease (CVD) is currently a leading cause of morbidity and mortality in the Western world, and is projected to remain so over the next 20 years¹⁹⁹. This fact has provided a strong impetus for assessing endothelial function *in-vivo*, and as a result several methods have recently emerged and are currently being used. These methods are diverse, each looks at a different vascular bed, and there appears to be very little uniformity among investigators regarding protocol. The aim of this review is threefold. First, to briefly review current knowledge of normal vascular endothelial physiology; second, to critically review the methods currently being used for peripheral and coronary endothelial function testing *in-vivo*, giving particular emphasis to those that are non-invasive or moderately invasive; and third, to examine how these methods have been applied for the study of endothelial dysfunction in response to risk factors of vascular disease.

3.2 Search Methodology

Literature search for this review was conducted on-line by the lead researcher (myself) using Medline, accessed via www.PubMed.gov. The following keywords and key phrases were used: vascular, microvascular, endothelium, endothelial cell, endothelial dysfunction, smooth muscle, nitric oxide, flow-mediated dilatation, FMD, laser Doppler iontophoresis, laser Doppler flowmetry, venous occlusion plethysmography, VOP, pulse wave velocity, augmentation index, applanation tonometry, diabetes mellitus, hypertension, cigarette smoking, hypercholesterolaemia, oestrogen, menopause, age.

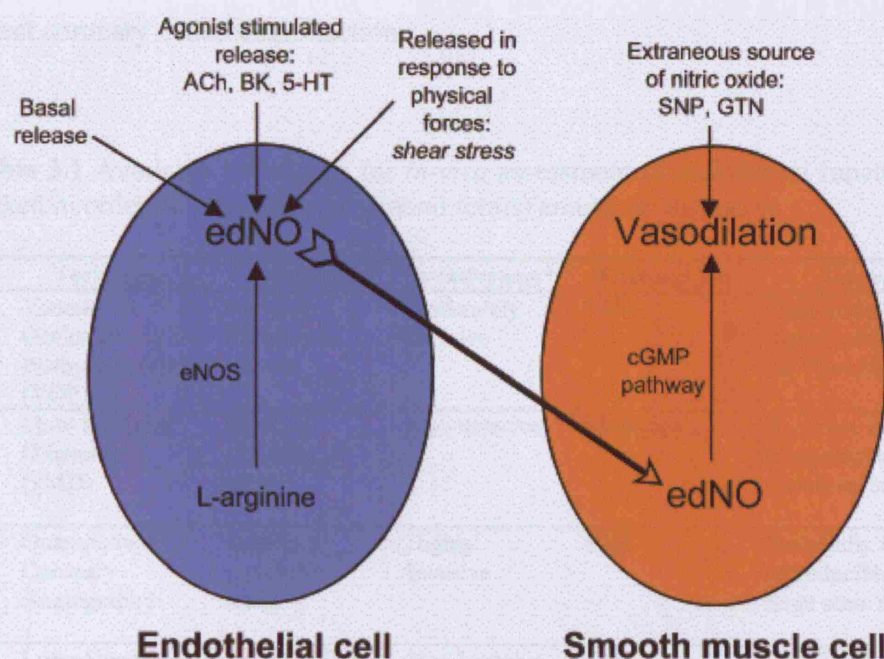
3.3 Physiology of Endothelial Function

The vascular endothelium can be regarded as a cellular monolayer that exists between the vessel lumen and vascular smooth muscle cells, in a layer designated the *tunica intima*. In essence it is a highly active paracrine organ, producing a variety of vasoactive molecules that control and regulate vasomotor tone. Of critical importance is endothelium-derived nitric oxide (edNO), previously known as endothelium-derived relaxing factor⁴⁵. edNO is synthesised from the amino acid L-arginine by the endothelial isoform of nitric oxide synthase (eNOS). It has a very short half-life, being rapidly oxidised to nitrate and excreted in the urine. Newly synthesised edNO diffuses across the endothelial cell membrane to enter vascular smooth muscle cells, where it activates the guanylate cyclase pathway. This results in an increased intracellular concentration of the second messenger cyclic guanosine-3',5-monophosphate (cGMP), which mediates many of edNO's effects. edNO is a potent vasodilator, and the balance that it strikes with various endothelium derived vasoconstrictors and the sympathetic nervous system, is essential in the

maintenance of vascular homeostasis. In addition, edNO suppresses platelet aggregation, leucocyte migration and cellular adhesion to the endothelium. It is postulated that endothelial injury and dysfunction, leading to impaired synthesis and release of edNO and other mediators, is a critical step in the evolution of all vascular diseases¹⁹⁵⁻¹⁹⁸.

There are several mechanisms which can induce and sustain the production of edNO (Figure 3.1). Firstly, there is a continuous basal release of edNO, as demonstrated by use of the antagonist L-arginine analogue *N*^G-monomethyl-L-arginine (L-NMMA). Infusion of L-NMMA into the human forearm via the brachial artery causes a rise in local arterial pressure, which can be reversed by administering L-arginine. Basal release of edNO is critical in the maintenance of resting vascular tone in resistance vessels, but is not so significant in conduit arteries (such as the brachial or coronary). Secondly, edNO production can surge under the stimulation of certain chemical substances, such as acetylcholine, bradykinin, serotonin and substance P. These substances bind to receptors on the endothelial cell membrane and induce production of edNO, thereby causing “endothelium-dependent vasodilatation”. Nitric oxide donors, such as sodium nitroprusside, can bypass endothelial cells and deliver nitric oxide directly to vascular smooth muscle cells, thus demonstrating “endothelium-independent vasodilatation”. Lastly, physical forces can induce the release of edNO. The viscous drag of flowing blood causes a haemodynamic *shear stress*, which is important in the physiological regulation of edNO. Shear stress induced edNO release is thought to involve three mechanisms, activated when there is a sudden increase in blood flow. First, there is an extremely rapid influx of calcium ions into the endothelial cell, activating the eNOS pathway and thereby increasing edNO output. Second, there is a rapid mechanical

Figure 3.1 Diagram summarising the physiology of endothelial function.



Key:

edNO – Endothelium-derived nitric oxide

eNOS – Endothelial nitric oxide synthase

ACh – Acetyl choline

BK – Bradykinin

5-HT – 5-hydroxy triptamine

SNP – Sodium nitroprusside

GTN – Glycerol trinitrate

cGMP – Cyclic guanosine monophosphate

activation of eNOS induced by the shear stress itself. Third, there is a slower response to shear stress by way of increased eNOS gene transcription sustaining a long term production of edNO^{45;198;200;201}.

The methods currently available for the detection of endothelial function *in-vivo* are listed in Table 3.1. For the purposes of this review, these methods will be divided into

those that detect endothelial function in various peripheral vascular beds, and those that detect coronary endothelial function.

Table 3.1 Available techniques for *in-vivo* assessment of endothelial function. They are ranked in order of popularity (in general terms) among investigators.

	Technique	Vascular Bed	Invasiveness*	Relative Cost	Accuracy/Reproducibility
1	Venous Occlusion Plethysmography (VOP)	Forearm Resistance Vessels	Moderately Invasive	Low	Claimed to be highly accurate and reproducible. Regarded by some as the “Gold Standard”.
2	Flow-Mediated Dilatation (FMD)	Brachial (conduit) artery	Non-Invasive	Moderate	Accuracy depends on quality of equipment/software. Observer variations are an issue.
3	Quantitative Coronary Angiography	Coronary (conduit) artery	Highly Invasive	High	Potentially highly accurate and reproducible. Invasiveness hinders large scale studies.
4	Laser Doppler Iontophoresis	Forearm Skin Microvessels	Non-Invasive	Low	Conflicting reports. No clear data as yet.
5	Pulse Wave Analysis	“Global” Endothelial Function	Non-Invasive	Low	Unclear how accurately global arterial stiffness reflects endothelial function.
6	Non-Invasive VOP	Forearm Resistance Vessels	Non-Invasive	Low	As accurate as standard VOP, but not widely used.
7	Magnetic Resonance FMD	Brachial (conduit) artery	Non-Invasive	High	Accurate, with better reproducibility than standard FMD.
8	CPT/MBF with PET**	Myocardium	Non-Invasive	High	Not yet assessed.
9	Retinal Arterial Architecture	Retinal arterioles	Non-Invasive	Moderate	Not yet assessed.
10	Flow-mediated changes in pulse wave velocity	Brachial to radial (conduit) artery	Non-Invasive	Moderate	One study only, but claims greater sensitivity to endothelial impairment than standard FMD technique.

* Procedures requiring intra-venous access only are regarded as non-invasive

** Cold Pressor Testing/Myocardial Blood Flow with Positron Emission Tomography

3.4 Assessing Peripheral Endothelial Function *In-Vivo*

3.4a. Venous Occlusion Plethysmography

Venous Occlusion Plethysmography (VOP) has been used to study forearm blood flow for many years. In fact, it was first described in 1909 by Hewlett and van Zwaluwenburg²⁰², and aside from the incorporation of computer technology, the method has remained essentially unchanged since then. The underlying principle involves the arrest of venous outflow from the forearm such that it begins to swell. The rate and degree of swelling reflects forearm vascular resistance, which in essence is purely a function of arterial vasomotor tone. Since an intact vascular endothelium is critical in maintaining normal arterial tone, this technique allows a direct assessment endothelial function within the forearm, and by inference, the rest of the arterial tree.

The method is widely used, with most practitioners using the protocol established by Wilkinson and Webb²⁰³. When performing the procedure it is critical that baseline vasomotor tone remains constant, so factors which may affect vasoreactivity are carefully avoided. Thus, VOP is performed in a quiet, temperature-controlled room, with the subject relaxing in a reclining position. Subjects are asked to abstain from fatty meals, alcohol, caffeine and tobacco in the preceding six hours (depending on the requirements of the study being undertaken). Blood pressure cuffs are placed around the arm and around the wrist, and the arm is held above the level of the heart. The arm cuff is inflated just enough to occlude venous outflow while preserving arterial inflow (40 mmHg), and the hand is excluded from the circulation by inflating the wrist cuff to supra-systolic pressures (200 mmHg). As the isolated forearm begins to swell, forearm volume increases in direct proportion to forearm blood flow. Forearm volume is measured by

means of a voltage dependent strain-gauge, placed circumferentially one third of the way down the forearm. The hand is excluded because it's blood flow is highly temperature sensitive, and it contains a high proportion of arterio-venous shunts. A degree of hand ischaemia is inevitable, and limits the testing period to no more than 10 minutes. Both arms are usually studied at the same time, the contralateral arm providing a contemporaneous control²⁰³. The increase in forearm volume is taken to represent blood flow in the *resistance vessels* of the forearm (muscle, soft tissues and skin).

VOP provides a convenient platform for testing forearm vascular resistance. In turn, vascular resistance can be readily manipulated by administering vasoactive drugs, most commonly acetylcholine but also bradykinin, serotonin, or in fact any other agent that is safe for use, directly into the brachial artery using a small (27 gauge) cannula. Changes in vascular resistance reflect endothelial function, and can be compared to the effect of agents such as sodium nitroprusside, which causes endothelium-independent vasodilatation. Investigating pharmacological effects in a closed circuit in this fashion has the advantage of avoiding systemic infusions of potentially dangerous drugs, and although there is a theoretical risk of critical forearm ischaemia when investigating vasoconstrictors such as endothelin, this is seldom seen in practice. Complications arising from repeated brachial arterial cannulation are rare²⁰⁴, nevertheless the procedure should be regarded as moderately invasive. In light of this, some investigators have developed a non-invasive variant of the VOP technique²⁰⁵⁻²⁰⁷. This involves using the principle of *reactive hyperaemia* employed in the brachial artery flow-mediated dilatation technique described below. This provides a non-invasive flow stimulus, thus avoiding the need for

arterial cannulation. Clearly, this adaptation negates its use as a pharmacological research tool, but it may prove a useful alternative for endothelial function testing.

Over the years, VOP has proved itself to be a robust and reliable tool for the investigation of vascular function. Despite an image of being cumbersome and somewhat dated, the procedure is well tolerated and continues to be popular. Using intra-arterial infusions of acetylcholine and sodium nitroprusside, VOP has been used to associate endothelial dysfunction with a range of cardiovascular risk factors such as smoking, hypertension, diabetes and aging²⁰⁸⁻²¹³. Most authors have found good reproducibility^{214;215}, and some even regard the technique as the “gold-standard” for the assessment of vascular function *in-vivo*^{203;216}.

3.4b. Brachial Artery Flow-Mediated Dilatation

Blood vessels have the capacity to adjust blood flow in response to luminal physical and chemical stimuli. This ability to self-regulate vasomotor tone allows the vessel to respond to changes in the local environment. As outlined above, an increase in blood flow will result in an increase in the *shear stress* to which the local vascular endothelium is subjected. This causes a temporary surge in edNO production which causes local arterial dilatation, a phenomenon called flow-mediated dilatation (FMD)^{45;198;200;201}. Using a high resolution ultrasound scanner, it is possible to monitor and record changes in vessel diameter resulting from FMD, and thus non-invasively determine endothelial function.

FMD is measured in conduit arteries, usually of a diameter between 2.5mm and 5mm, and the brachial artery is a convenient choice. The size of the vessel is an important consideration, if the vessel is too small then it will be technically difficult to obtain

accurate and reproducible images, whereas if too big then vasodilatation may be difficult to perceive, even when endothelial function is normal²¹⁷. A high resolution ultrasound scanner with a high frequency vascular transducer and an internal ECG monitor is required. The scanner must be equipped with appropriate vascular software for 2-D imaging, colour and spectral Doppler. Subjects are examined supine, with the arm held in a comfortable position, and care must be taken to avoid vasoreactive factors (see above). The brachial artery is examined in the longitudinal plane above the antecubital fossa. It is essential that a steady image of the artery is maintained throughout the study, and the relative position of anatomic landmarks such as veins and fascial planes are noted²¹⁸.

A blood pressure cuff is used to create the flow stimulus in the brachial artery. The cuff is placed either above or below the transducer position. When placed above, the stimulus is greater, but accurate visualisation is more difficult. After a baseline measurement, the cuff is inflated to a suprasystolic pressure for up to 5 minutes. Cuff deflation induces a brief high flow state (reactive hyperaemia) which increases the shear stress on the brachial artery, and results in endothelium-dependent vasodilatation. FMD is monitored from 30s before to 2 minutes after cuff deflation. The image should return to the baseline after a ten min rest period. At this point, 500 micrograms sublingual glyceryl trinitrate (GTN) is administered to assess endothelium-independent vasodilatation^{196;197}, which is theoretically the maximum vasodilator response.

While some investigators have found the technique to be both accurate and reproducible¹⁹⁸, others have not been so impressed^{199;200}. There is no doubt that FMD, like any other operator dependent procedure, is vulnerable to criticisms of poor reproducibility and intra/inter-observer variability. An International Brachial Artery

Reactivity Task Force has been appointed, and steps to investigate and minimise these problems are underway¹⁹⁷. Ultrasonographic assessment of the brachial artery is uniquely challenging and the examination must be performed by well trained, skilled individuals. There is a significant learning curve, and once sufficiently trained, sonographers must continue to perform the technique on a regular basis to maintain their skills. Ergonomic factors are also important, with both examiner and subject assuming comfortable positions, such that the transducer remains steady at the same anatomical position throughout. This may seem straightforward, but is actually quite a technical challenge, and the use of a stereotactic probe holding device has been advocated^{196;197} (and should probably be considered mandatory). Even when excellent images of the brachial artery are obtained, off-line diameter measurement using ultrasound calipers is not reliable, and should be regarded as out of date. Most investigators now use radio-frequency processing software, as this facilitates accurate on-line tracking of the anterior and posterior arterial walls¹⁹⁹. To account for diameter changes associated with the cardiac cycle, the signal must be ECG gated. Systems of this type can give a theoretical accuracy of the order of 5 microns, although this is highly dependent on the quality of the image obtained. Most importantly however, these systems are automated so they take the responsibility of measuring diameter away from the operator thus theoretically reducing the rate of observer error.

The theory and methodology behind FMD measurement is now well established, and most vascular laboratories are already equipped with the necessary hardware. One can anticipate improvements in the technique in parallel with advances in imaging technology and computer software; for example, phase-contrast magnetic resonance

angiography has been advocated as an alternative to high resolution ultrasound^{201;202}. Although this may prove superior in detecting minute vessel diameter changes, at present ultrasound is preferred as it is more dynamic (and cheaper). Another variation on the technique combines the principles of FMD with those of non-invasive pulse wave analysis described below. In essence, the authors measure variations in pulse wave velocity from brachial to radial artery (or brachial to ankle) in response to stimuli such as post-occlusive hyperaemia or acetylcholine. They claim a greater sensitivity to endothelial dysfunction than standard brachial artery diameter measurements in a study comparing 17 healthy controls to 7 chronic heart failure patients²¹⁹. But this interesting adaptation of the FMD principles is very new and as yet no other group has tried it.

3.4c. Laser Doppler Iontophoresis

Iontophoresis is a convenient and simple technique for transdermal administration of a minute quantity of a drug, using electric currents. The principle is that the molecules of a drug in solution are either positively or negatively charged, and will migrate across the skin under the influence of an applied direct monopolar current²²⁰. The rate and quantity of drug delivered is dependent on the magnitude of the current and it's duration²²¹:

$$\text{charge (Coulombs)} = \text{current (Amps)} \times \text{time (seconds)}$$

This method of drug delivery is not new, and has been in use for over 20 years²²². However, it has now become apparent that, when used in conjunction with laser Doppler flowmetry, it is a useful tool for monitoring microvascular endothelial function²²³. When used in this way, this technique is known as laser Doppler iontophoresis (LDI).

The procedure involves time-controlled delivery of a vasoactive drug onto a patch of skin on the subjects forearm. The resultant alterations in skin blood flow are then detected using a laser Doppler flowmeter, or more recently using a laser Doppler imager²²⁰. Laser Doppler imaging allows blood flow measurement over the entire distribution of the administered drug, whereas laser Doppler flowmetry restricts blood flow measurements to single points in the distribution. The alterations in blood flow reflect endothelial function at a microvascular level. Acetylcholine and sodium nitroprusside are used to generate “endothelium-dependent” and “endothelium-independent” vasodilatation respectively. The procedure is carried out using very small currents (less than 100 microAmps), and is painless. The forearm microvascular bed is usually the site of choice, and iontophoretic electrodes are attached to the volar aspect. The quantity of drug delivered is too small to have any systemic effects, although mild allergic reactions and skin irritation have been reported²²⁴.

LDI is an attractive technique, but there are several important problems that must be overcome. It could be argued that the variability of skin conductivity in different populations should be considered when designing studies and interpreting results. While Ohm’s Law has been used to correct for these differences, one could argue that applying such physical laws to organic tissue adds a spurious air of accuracy. Although the procedure is short (typically 15 minutes), subjects are required to remain entirely still during that time, with the forearm held in supination; this may result in movement artefacts owing to subject fatigue.

However, by far the most significant problem is the tendency of the applied current itself to cause vasodilatation, even in the absence of an administered drug. The

precise aetiology of *current-induced vasodilatation* is complex and has yet to be fully defined. It has been well documented that current-induced vasodilatation is more pronounced with cathodal (acetylcholine) rather than anodal (sodium nitroprusside) drug delivery²²⁴⁻²²⁸. Some investigators have postulated that primary afferent nerves, specifically C-nociceptive fibres, may have an important role^{225;228}. Neurovascular responses are thought to be directly stimulated by the applied current, resulting in the release of vasodilatory neuropeptides, such as calcitonin gene-related peptide and substance P. This neural stimulation may also release various aspirin sensitive mediators that contribute to the vasodilatory effect^{226;229}.

In an attempt to minimise the effect of current-induced vasodilatation, the use of topical lignocaine cream has been advocated^{224;227}, but it is unclear whether lignocaine abolishes the specific neural pathway responsible for the vasodilatory effect. Oral aspirin has been tried with more success^{226;229}, as has use of a low resistance solution vehicle for drug delivery, such as 0.5% NaCl²²⁷. Nevertheless, the most acceptable way of limiting current-induced vasodilatation seems to be the use of smaller currents over longer time-periods. The total charge (and drug dose) delivered remains the same, but stimulation of the neurovascular response is less pronounced.

LDI is becoming increasingly popular, and with good reason. It is non-invasive, provides a direct assessment of microvascular endothelial function, and is relatively resistant to accusations of observer dependency. Reproducibility can be improved if precautions such as strict standardization of the recording site are taken²³⁰, however, the procedure is better for determining differences in groups rather than individuals.

3.4d. Pulse-Wave Analysis

LDI and FMD detect endothelial dysfunction in local vascular beds, either skin microvasculature or the brachial arterial tree. Pulse-wave analysis is a relatively new technique that provides a non-invasive method of assessing global endothelial function^{231;232}. Arterial stiffness is partly dependent on vasomotor tone²³³, which in turn relies on an intact endothelium. As the arterial pulse waveform travels from the central circulation to the periphery, its shape provides a measure of systemic arterial stiffness²³⁴, thus changes in the shape of the waveform will partly reflect endothelial function. The waveform is readily analysed at the radial artery by applanation tonometry. This involves the placement of a small probe over the radial artery at its maximal point of pulsation, such that the arterial wall is slightly flattened, creating circumferential stress; removal of the probe releases this stress. The resulting changes in intra-luminal pressure are mirrored by changes in the electrical resistance of a small piezoelectric crystal within the tip of the probe, and this is translated into a waveform²³¹.

The arterial pulse waveform is reflected from the periphery back to the central circulation²³⁵, and this may confound the observed radial waveform. To account for this, endothelial function is assessed by changes in the Augmentation Index (AIx). AIx is a function of the relationship between the reflected arterial wave and the primary aortic wave²³⁵⁻²³⁷. It is calculated as the ratio of the pulse pressure at the second systolic peak to that at the first systolic peak²³¹. In essence, AIx provides a measure of systemic arterial stiffness, and several studies have used it as a marker for endothelial function^{231;232;238;239}.

Endothelium-dependent vasodilatation is assessed following inhalation of a B₂ agonist (salbutamol), which has been shown to cause the release of edNO²⁴⁰. This is then

confirmed by administering the eNOS antagonist N^G -monomethyl-L-arginine (L-NMMA), which blocks this effect. Endothelium-independent vasodilatation is assessed following intravenous administration of GTN. After baseline measurements of blood pressure and heart rate, the probe is placed at the wrist and the appropriate drug administered by an infusion pump. Serial recordings of the pulse waveform are taken over the next 20 min, and the information uploaded onto waveform analysis software.

The technology required for PWA is easily available and relatively cheap. Unlike FMD, the procedure is quite immune to criticisms of observer dependency. More work needs to be done to clarify whether AIx is a consistently reliable parameter. Those with high cardiovascular risk or established atherosclerotic disease may exhibit different wave reflection characteristics from the groups studied thus far. Similarly, baseline cardiac parameters may preclude certain patients from study. A high resting heart rate, for example, may have an independent association with arterial stiffness²⁴¹. In fact, the precise association between arterial stiffness and endothelial function is still unclear.

3.4e. Retinal Arterial Abnormalities

Abnormalities of architecture in an arterial network can reflect generalised circulatory efficiency. The retina has long provided a convenient arterial bed in which it is possible to make non-invasive, *in-vivo* assessments of arterial architecture. The presence of abnormalities, such as focal and generalised narrowing, arteriovenous nicking, altered arteriole to venule ratio, and sub-optimal arterial diameter at bifurcations, can be suggestive of a more generalised circulatory disorder²⁴²⁻²⁴⁴. It has been reported, most notably by the Atherosclerosis Risk in Communities (ARIC) Study, that abnormalities in

retinal arterioles are related to the presence of carotid plaque, hypertension, and serum markers of inflammation and endothelial function such as von Willebrand Factor^{242;243}. Although it has been reported that inhibition of NO synthase by L-NMMA induces changes in arteriolar junctions and bifurcations in human retinal arteries²⁴⁵, a precise correlation between peripheral endothelial dysfunction and retinal arterial abnormalities has yet to be made. A correlation has, however, been made between the presence of peripheral vascular disease and retinal arteriolar abnormalities²⁴⁴.

The retinal arteriolar network can be assessed using non-invasive scans, which are then assessed by trained individuals. The notion that a simple retinal scan can provide information about vascular function is enticing. However, while it is quite likely that established cardiovascular disease with co-existent widespread haemodynamic impairment can be reflected in retinal arterial architecture, no group has yet demonstrated that generalised endothelial dysfunction *per se* can result in retinal arterial abnormalities.

3.5 Assessing Coronary Endothelial Function *In-Vivo*

3.5a. Quantitative Coronary Angiography

The techniques described above have been used to assess the impact of cardiovascular risk factors on endothelial function in peripheral vascular beds. Coronary angiography, however, provides a means of directly assessing coronary endothelial function²⁴⁶. The principles of endothelial function testing are the same as described above. Access to the coronary circulation is achieved via the femoral artery, through which a cardiac catheter is passed²⁴⁷. Vasoactive substances, such as acetylcholine or substance P are instilled directly into the coronary circulation, stimulating endothelium-dependent vasodilatation,

and glyceryl trinitrate can be used to stimulate endothelium-independent vasodilatation²⁴⁸⁻²⁵². Coronary angiograms depicting changes in vessel diameter are recorded and analysed off-line. Coronary blood flow is assessed by use of a Doppler probe passed into the coronary artery at the region of study. Intravascular ultrasound (IVUS) may be incorporated into the study protocol, allowing assessment of coronary artery wall thickness and established coronary artery plaque^{120;253}.

The procedure is highly invasive, and carries substantially more risk than the other procedures described, despite widespread use and expertise. And it must be borne in mind that introducing vasoactive pharmacological agents into the coronary circulation is itself not without risk. Where patients are scheduled to have a coronary angiogram on clinical grounds, it is perhaps easier to justify coronary endothelial testing. Thus, it has proved useful in the assessment of risk factor modification, for example with lipid-lowering agents, in patients with established CAD or transplant recipients^{120;254}. However, the procedure is not attractive as a means of assessing the affect of cardiovascular risk factors on the coronary circulation in otherwise healthy individuals.

3.5b. Cold Pressor Testing and Myocardial Blood Flow

Studies have shown a correlation between increased coronary blood flow observed during intra-arterial administration of the endothelium dependent vasodilator acetylcholine, and the increase in myocardial blood flow (MBF) observed after cold pressor testing (CPT)²⁵⁵. This increase in MBF is thought to be due to the principles of flow-mediated dilatation described earlier. The underlying principle involves nociceptor driven sympathoadrenergic stimulation following, for example, immersion of an extremity in

ice-cold water. This results in an increase in myocardial oxygen demand, and thus MBF^{255,256}. Positron emission tomography (PET) using the tracer ¹³N-ammonia, provides a non-invasive means of assessing MBF, and thus myocardial endothelial function²⁵⁶⁻²⁶⁰.

Measurements are conducted in similar baseline conditions to those described above. A precise account of the methodology of this technique is beyond the scope of this review. Briefly, the subject's foot and ankle are immersed in ice-cold water for 2 to 3 minutes, during which time intravenous tracer is administered, and serial PET scans performed. To acquire an accurate series of images, the subject remains motionless, and scans performed at precisely the same anatomical point. Computer aided image analysis is carried out afterwards, with "regions of interest" being identified by trained observers.

Cold pressor testing does increase MBF, but even in normal subjects, the effect can be quite small, or even non-existent²⁵⁶. The pharmacologic agent dipyridamole is used to assess maximum MBF, as it acts as a myocardial stressor, and thus increases myocardial oxygen demand directly²⁵⁶⁻²⁶⁰. Use of PET scanning to observe MBF should be accurate, as long as steps are taken to minimise errors in image interpretation. Motion artefacts may be a particular problem, especially in view of the pain and discomfort associated with CPT. Unfortunately, given the expense and limited availability of the equipment, these issues are likely to remain unclarified for some time to come. Nevertheless, there is no doubt that a non-invasive technique that allows direct assessment of coronary endothelial function is compelling. Interestingly, some investigators have used trans-thoracic echocardiography with "microbubbles" for the assessment of MBF²⁶¹. As far as we are aware, this technology has not yet been applied for myocardial endothelial function testing.

3.6 Endothelial Dysfunction In Vascular Pathogenesis

3.6a. Diabetes Mellitus

Diabetes mellitus is a well recognised cause of macro- and micro-vascular disease. It is postulated that chronic hyperglycaemia has a detrimental effect on long term endothelial function, resulting in accelerated atherosclerosis and widespread microvascular disease. However, the precise mechanism is not yet clear and may involve several processes, such as altered anti-oxidant and lipid metabolism, insulin resistance, and dysregulation of ET-1^{213;262-265}. Acute hyperglycaemic episodes can also have direct and reversible effects on endothelial function, as demonstrated by observations in non-diabetics with a degree of glucose intolerance, and in gestational diabetes^{266;267}.

The global nature of vascular disease associated with diabetes has made it a useful “testbed” for some of the procedures described above. VOP, FMD and LDI have all been used extensively to study the effects of diabetes on endothelial function, and have proved useful in assessing the endothelial response to risk factor modifying treatment (Table 3.2). Reliable *in-vivo* assessment of endothelial function could in theory prove useful in a clinical setting. In addition to providing a means of monitoring the evolution of vascular disease, it could provide an additional means of assessing the adequacy of long term glycaemic control, and allow therapeutic strategies to be targeted accordingly. At present, none of the techniques described above demonstrate sufficiently accurate reproducibility to make this feasible in individuals, although they are useful for monitoring groups.

Table 3.2. Assessment of peripheral endothelial function in diabetes mellitus..

Author	Method	Number	Hypothesis	Outcome
Van de Ree et al. ²¹³	VOP	17 IDDM 10 HC	EF is impaired in diabetes.	Vasodilator response to serotonin was significantly lower among diabetics than controls (p<0.05).
Mather et al. ²⁶⁴	VOP	28 metformin 15 placebo	Reducing insulin resistance improves EF.	Significant improvement in ACh mediated blood flow in metformin group (p=0.0012).
Cheetham et al. ²⁶⁵	VOP	9 IDDM	Losartan improves EF in diabetics.	Losartan significantly reduced forearm vascular resistance in response to ACh (p<0.05).
Timimi et al. ²⁶⁸	VOP	10 IDDM 10 HC	Anti-oxidants (Vit C) improve EF.	Forearm blood flow in response to methacholine augmented by vit C administration (p=0.001).
Playford et al. ²⁶⁹	FMD	17 fenofibrate 18 HC	Fenofibrate improves EF.	Fenofibrate significantly improved FMD% over placebo by 1.48% (p=0.01).
Ravikumar et al. ²⁷⁰	FMD	50 IDDM 50 HC	EF is impaired in diabetes.	FMD% significantly impaired in diabetics vs. control (2.9 vs. 4.4, p=0.0001).
Anastasiou et al. ²⁶⁷	FMD	33 previous GD 17 HC	EF is impaired in recovered gestational diabetes.	FMD% significantly impaired in study vs. control (1.6 vs. 10.3, p<0.001).
Lekakis et al. ²⁷¹	FMD	26 IDDM 26 HC	Diabetics with and without micro-albuminuria have impaired EF.	FMD% 0.75 and 5.8 vs. 11 (p=0.003).
Khan et al. ²⁷²	LDI	56 IDDM 22 HC	EF is impaired in type 1 diabetics.	Skin flux response to ACh significantly impaired in IDDM vs. control (p<0.01).
Caballero et al. ²⁷³	LDI	30 HC 39 family history	EF is impaired in non-diabetics with a positive family history.	ACh and SNP responses attenuated in study group vs. control (p<0.001).
Katz et al. ²⁷⁴	LDI	19 IDDM 10 HC	Impaired non-endothelium dependent flow is also significant.	Skin blood flow increased 20-fold in controls, and was attenuated by 18% in diabetics (p<0.05).
Wilkinson et al. ²³⁸	PWA	35 IDDM 35 HC	Arterial stiffness parameters are elevated in type 1 diabetics.	Significantly lower augmentation index in IDDM over control (p<0.01).

Key :

VOP-Venous Occlusion Plethysmography; FMD-Flow-Mediated Dilatation; LDI-Laser Doppler Iontophoresis; EF-Endothelial Function; IDDM-Insulin Dependent Diabetes Mellitus; HC-Healthy Controls.

3.6b. Essential Hypertension and Endothelial Function

Essential hypertension is associated with a generalised impairment of endothelial function, resulting in a system-wide increase in vasomotor tone and vascular resistance. The underlying cause is not known, but may involve oxidative stress^{275;276}, endothelin overactivity²⁷⁷, genetic/racial factors²⁷⁸ and primary alterations in the structure and function of microvessels²⁷⁹⁻²⁸¹. Alterations in microvascular structure and function underlies the end-organ damage seen in chronic hypertensives, but the aetiology of microvascular injury is unclear. It may occur secondary to prolonged systemic hypertension, in which case there must be a primary systemic endothelial defect. Or it may itself be the primary event, raising systemic vascular resistance, with subsequent haemodynamic changes leading to secondary endothelial damage, and ultimately atherosclerosis²⁸⁰⁻²⁸².

Several studies have demonstrated impaired *in-vivo* endothelial function in hypertensives (Table 3.3), utilising VOP, FMD and LDI. At least two groups have demonstrated improvement in endothelial function after anti-oxidant therapy, supporting a role for reactive oxygen species in disease pathogenesis^{275;276}. Interestingly, FMD testing has shed new light on the association between hypertension and obesity; evidence is emerging that the latter has an independent effect on endothelial function²⁸³.

Table 3.3. Hypertension and endothelial function.

Author	Method	Number	Hypothesis	Outcome
Sasaki et al. ²¹⁰	VOP	11 subjects 15 controls	Low calorie diet improves EF in obese hypertensives.	Forearm blood flow in response to ACh improves after weight loss ($p<0.05$).
Perticone et al. ²⁸⁴	VOP	225 never treated hypertensives	Impaired EF has prognostic significance in hypertension.	Forearm blood flow in response to ACh is a marker for future cardiovascular events ($p<0.005$).
Taddei et al. ²⁷⁶	VOP	14 hypertensives 14 normotensives	Vit C improves EF in hypertension.	Significant improvement in forearm blood flow ($p<0.01$).
Modena et al. ²⁸³	FMD	400 PMW with hypertension	Improvements in EF in PMW on anti-hypertensive treatment have prognostic significance.	At six months 250 showed improvement in EF (FMD> 10%), and this group suffered fewer cardiac events ($p<0.0001$).
Duffy et al. ²⁷⁵	FMD	39 subjects 82 controls	Vit C improves EF in hypertension.	No improvement in FMD% or GTN%.
Pierdomenico et al. ²⁸⁶	FMD	22 hypertensives 22 normotensives	EF is worse in sustained compared to “white coat” hypertension.	FMD% is significantly lower in sustained hypertension (4.5% vs. 7.5%, $p<0.05$).
Irving et al. ²⁸⁷	LDI	105 healthy young men	Higher blood pressure is linked with impaired EF.	Dermal flux in response to ACh not correlated to blood pressure.

Key :

VOP-Venous Occlusion Plethysmography

FMD-Flow-Mediated Dilatation

LDI-Laser Doppler Iontophoresis

EF-Endothelial Function

PMW- Post-Menopausal Women

3.6c. Endothelial Dysfunction and Cigarette Smoking

Smoking is well established as a risk factor for cardiovascular disease, and constitutes a major public health concern. Cigarette smoke contains many chemical compounds that are potentially toxic to organic tissue, so it is difficult to establish a precise mechanism of vascular injury²⁸⁸. Regular smoking results in accelerated atherosclerosis of peripheral and coronary arteries, loss of vasomotor tone, and a generalised stiffening of the arterial tree. These effects are principally the result of sustained endothelial dysfunction, although direct smooth muscle impairment, and alterations in coagulation state are important^{289;290}. It is thought that toxins in cigarette smoke induce endothelial production of oxygen-derived free radicals, such as the superoxide anion. The resulting endothelial injury reduces bioavailability and inactivation of edNO²⁹¹. In addition, smoking may impair important endocrine mechanisms that are involved in the maintenance of vasomotor tone, such as the renin-angiotensin system²⁰⁹.

The methods described in this review have been used to establish an association between cigarette smoking and endothelial dysfunction in most vascular beds (Table 3.4). This effect is present in long-term smokers, but short-term smokers, and even those who have smoked just a single cigarette, can have demonstrable endothelial impairment²⁹².

Table 3.4 Assessment of peripheral endothelial function in cigarette smokers.

Author	Method	Number	Hypothesis	Outcome
Butler et al. ²⁹⁸	VOP	29 smokers 39 non-smoker	EF is impaired in smokers	Significant blunting of flow ratio between smokers and non-smokers using ACh (3.4 vs. 4.0, $p=0.04$).
McVeigh et al. ²⁹³	VOP	35 smokers 16 non-smokers	EF is impaired in smokers	Significant impairment in basal blood flow only (attenuated L-NMMA response), $p<0.01$.
O'Grady et al. ²⁹⁴	FMD	10 smokers 10 non-smokers	EF is impaired in smokers.	FMD% smokers vs. non-smokers was 2.6% vs. 9.5% respectively ($p<0.005$).
Barua et al. ²⁹⁵	FMD	7 light smokers 8 heavy smokers	EF is worse in heavy compared to light smokers.	No significant difference in FMD% between light smokers vs. heavy smokers (1.0% vs. 0.1%).
Neunteufl et al. ²⁹⁶	FMD	16 healthy smokers	Nicotine impairs EF in smokers.	Smoking and nicotine spray caused FMD% reduction of 10.2-6.7 and 9.2-3.8 ($p<0.0001$).
Ijzerman et al. ²⁹²	LDI	12 young healthy smokers	Smoking affects microvascular EF acutely	Skin response to ACh attenuated after smoking ($p=0.04$).
Pellaton et al. ²⁹⁷	LDI	20 young smokers 20 older smokers	EF in older smokers is worse than in younger.	Skin response to ACh attenuated in older vs. younger smokers ($p<0.05$).
Mahmud et al. ²⁹⁸	PWA	41 smokers 116 non-smokers	Arterial stiffness is increased in smokers.	Augmentation index higher in chronic than non-smokers (0.7 vs. -5.7, $p<0.01$).

Key :

VOP-Venous Occlusion Plethysmography

FMD-Flow-Mediated Dilatation

LDI-Laser Doppler Iontophoresis

EF-Endothelial Function

PWA-Pulse Wave Analysis

3.6d. Endothelial Dysfunction and Hypercholesterolaemia

Hypercholesterolaemia is an independent risk factor for cardiovascular disease. LDL-cholesterol has a directly damaging effect on vascular endothelium through the production of oxygen-free radicals; HDL-cholesterol is thought to have antagonistic effects on LDL-cholesterol in this regard. Hypercholesterolaemia may also promote interaction between caveolin and eNOS, further reducing edNO²⁹⁹⁻³⁰¹.

It is clear that these effects are most relevant in sustained hypercholesterolaemia, however, acute lipid-loads (in the post-prandial state) can cause endothelial dysfunction in both conduit and forearm resistance vessels, although this is not a universal finding. In addition, treatment with cholesterol-lowering medication can reverse endothelial dysfunction, and this is related to a large extent on the type of drug used^{299;301-304}. The methods described in this review appear to lend themselves well to the investigation of lipid-induced endothelial dysfunction, but there is a fair degree of inconsistency in the findings (Table 3.5).

Table 3.5 Assessment of peripheral endothelial function in hypercholesterolaemia.

Author	Method	Number	Hypothesis	Outcome
Gudmundsson et al. ³⁰⁵	VOP	15 healthy subjects	High fat meal impairs EF in resistance vessels.	No significant difference between high and low fat meals.
Raitakari et al. ³⁰²	NIVOP	12 healthy subjects	High fat meal increases resting forearm blood flow.	Forearm blood flow (ml/min/100ml) rose from 1.4 to 2.4, and 1.9 to 2.7 (p<0.001).
Mercuro et al. ³⁰⁶	VOP	28 subjects randomised to statin and placebo	EF improves in hyperlipidaemic post-menopausal women with statins.	Increased effect of acetylcholine after statin (p<0.01).
Inoue et al. ²⁹⁹	VOP	40 randomised hyperlipidaemic subjects	EF is improved by fluvastatin compared to pravastatin.	Peak reactive hyperaemia improved after treatment (p<0.01).
De Jongh et al. ³⁰⁷	FMD	50 positive FH 19 controls	Simvastatin improves EF in children with familial hypercholesterolaemia.	Absolute increase in FMD% 3.9 vs. 1.2 (p<0.05).
Omori et al. ³⁰³	FMD	30 subjects randomised to statin and placebo	Single dose cerivastatin improves EF independent of lipid-lowering effect.	FMD% improves significantly at 3 hours post statin (p<0.001).
Lupattelli et al. ³⁰⁰	FMD	72 high HDL 35 low HDL	High HDL compared to low HDL have better EF.	FMD% 3.3 in low HDL and 5.1 in high HDL cholesterol (p<0.001).
Stein et al. ³⁰¹	FMD	37 randomised hyperlipidaemic subjects	Statins and anti-oxidants (Vit C) improve EF.	No significant change in FMD%.
Alonso et al. ³⁰⁴	FMD	25 subjects	Long term simvastatin improves EF in FH.	FMD% increase from 4.7 to 12.3 after 12 weeks (p<0.005).
Khan et al. ³⁰⁸	LDI	14 subjects	Statins improve EF in subjects with established peripheral arterial disease.	Significant improvement in SNP response (p<0.05) but not ACh.
Wilkinson et al. ²³⁹	PWA	68 subjects 68 controls	Arterial stiffness parameters are increased in hypercholesterolaemia.	Significant increase in augmentation index (p<0.001).

Key :

VOP-Venous Occlusion Plethysmography; NIVOP-Non-invasive VOP; FMD-Flow-Mediated Dilatation; LDI-Laser Doppler Iontophoresis; EF-Endothelial Function; PWA-Pulse Wave Analysis; ACh-Acetyl choline; SNP-Sodium nitroprusside

3.6e. The Effect of Age and Menopause on Endothelial Function

Age is an independent risk factor for cardiovascular disease, and the incidence of myocardial and cerebrovascular events is higher in the elderly population. Atherosclerotic disease becomes increasingly prevalent as age increases, and this is usually the result of long-term exposure to multiple risk factors. The specific effect of aging on the vascular endothelium is thought to be a progressive increase in oxidative stress, causing a reduction in edNO bioavailability. Evidence for this is provided by studies showing an improvement in endothelial function after administration of anti-oxidants, such as vitamin C^{211;276}. Others have postulated that aging has a negative effect on the L-arginine-NO pathway, perhaps involving an impairment of intra-cellular signal transduction³⁰⁹.

The effect of age is not uniform between the sexes, and the cardioprotective effect of oestrogen has been well documented; indeed post-menopausal women acquire a similar level of risk as men of the same age. Several mechanisms have been postulated to explain the cardioprotective effect of oestrogen. It has anti-oxidant properties³¹⁰, it causes the release of prostaglandins via cyclo-oxygenase dependent mechanisms^{311;312}, and it is said to directly promote eNOS synthesis via up-regulation of mRNA expression^{205;206}. Beneficial effects are not confined to the endothelium; it also has important effects on vascular smooth muscle cells and platelet aggregation³¹³. Administration of exogenous oestrogen causes an improvement in endothelial function in various vascular beds in post-menopausal women, and indeed in hypogonadal men also³¹⁴. However, these observations have not yet translated to clinical practice, and there is controversy as to the cardioprotective effects of hormone replacement therapy in post-menopausal women³¹⁵.

Interestingly, studies in pre-menopausal women have demonstrated a cyclical variation in endothelial function in parallel with menstruation, providing evidence that short term relative changes in the hormonal environment are significant³¹¹. This finding may have important implications for general study design when the subjects include pre-menopausal women. Studies assessing the effect of age and gender on endothelial function are numerous, and have been summarised in Table 3.6 and 3.7.

Table 3.6. Effect of age on endothelial function.

Author	Method	Number	Hypothesis	Outcome
Singh et al. ²¹²	VOP	18; mean age 32 15; mean age 65	Basal EF is impaired in the elderly.	Dose response curves of aspirin and L-NMMA significantly lower in older age group ($p<0.01$).
Singh et al. ²¹¹	VOP	18 healthy diet 18 placebo	“Mediterranean style” diet improves EF in the elderly.	Improved forearm blood flow in response to bradykinin ($p=0.043$).
De Souza et al. ³⁰⁹	VOP	68 subjects	Exercise restores EF in the elderly.	Forearm blood flow in response to ACh increases by 30% after training ($p<0.01$).
Lind et al. ³¹⁶	VOP	56; age 20-69	EF is related to age and other cardiovascular parameters.	Forearm blood flow in response to methacholine attenuates with age ($p<0.05$).
Rajagopalan et al. ³¹⁷	FMD	18; mean age 75	Losartan improves EF in the elderly.	No significant difference in FMD% from drug (3.2-2.4) to placebo (3-3.9).
Simons et al. ³¹⁸	FMD	20; age 45-70	Vit E improves EF in the elderly.	No significant change in FMD%.
Woo et al. ³¹⁹	FMD	19 Chinese 19 white	Elderly Chinese have better EF than whites.	FMD% significantly better in elderly Chinese (6.8% vs. 1.8%, $p<0.001$).
Rossi et al. ³²⁰	LDI	15; age >65 15; age <50	Aging is associated with impaired EF	Maximum ACh response similar, but dose-response lower in older group ($p<0.001$).
Pierzga et al. ³²¹	LDI	12; age <29 12; age >64	Aging is associated with impaired EF	Cutaneous vascular conductance in response to heating is impaired in older group ($p<0.05$).

Key :

VOP-Venous Occlusion Plethysmography

FMD-Flow-Mediated Dilatation

LDI-Laser Doppler Iontophoresis

EF-Endothelial Function

Table 3.7. Effect of menopause and oestrogen on endothelial function.

Author	Method	Number	Hypothesis	Outcome
Komesaroff et al. ³¹⁴	VOP	7 subjects 5 controls	Oestrogen improves EF in hypogonadal men.	Significant increase in forearm blood flow (p<0.01).
Chan et al. ³¹¹	VOP	15 healthy women (mean age 28)	EF changes during a normal menstrual cycle in otherwise healthy women.	Dose response to bradykinin increased at mid-cycle compared to early (p=0.01).
Sanada et al. ²⁰⁶	NIVOP	18 with HRT 15 without HRT	HRT improves EF in PMW.	Forearm blood flow improves in PMW with ID/II genotypes (30.6% to 32.6%, p<0.01).
Majumdar et al. ³¹³	VOP	20 subjects 12 controls	EF improves after HRT in PMW	Constrictor responses to L-NMMA increased after HRT (132 vs. 89, p<0.05).
Ohmichi et al. ³²²	FMD	12 subjects 8 controls	Surgical oophorectomy results in an acute deterioration in EF.	FMD% reduced from 9.4% to 4.2% pre- and post-op (p=0.0002).
Sorensen et al. ³²³	FMDMR	13 subjects 10 controls	Long-term use of depot contraception can cause deterioration in EF.	FMD% reduced in study group (1.1% vs. 8.0%, p<0.01).
Herrington et al. ³²⁴	FMD	1636 subjects, of which 291 are on HRT	HRT improves EF in PMW with established CVD.	Improvement in FMD% after HRT (p=0.01).
Calkin et al. ³¹²	LDI	32 healthy PMW.	COX inhibitors abolish oestrogen mediated improvements in EF	Significant increase in skin blood flow, but effect negated by celecoxib (p<0.05).
Arora et al. ³²⁵	LDI	20 pre-menopausal 9 PMW	EF is impaired in PMW compared to pre-menopausal.	Reduced response to ACh in PMW group (93% vs. 187%, p=0.001).

Key :

VOP-Venous Occlusion Plethysmography

NIVOP-Non-invasive VOP

FMD-Flow-Mediated Dilatation;

LDI-Laser Doppler Iontophoresis

EF-Endothelial Function

COX-Cyclooxygenase

PMW- post-menopausal women

3.7 Summary

Since the vascular endothelium exists uniformly throughout the arterial tree, it is reasonable to assume that endothelial function should also be uniform throughout the vascular tree. However, a particular problem appears to be a lack of consistency in the findings when one vascular bed is compared to another, and also when the same methods are compared. To a large extent, this is because of the relatively small numbers that most of these studies have generated. Another factor is that although these methods have been widely applied, some of the issues highlighted in this review (for example, using wall-tracking software with FMD, or avoiding current-induced vasodilatation with LDI) have not been universally addressed.

What are required are more comparative analyses, but so far these have been few and far between. The two most well established methods for assessing peripheral endothelial function are VOP and FMD. In a somewhat limited study of 6 healthy subjects compared to 10 hypertensive/obese individuals, Irace *et al.*³²⁶ reported a high correlation ($p < 0.001$) between these techniques. However, Lind *et al.*³²⁷ evaluated both these methods in 24 healthy subjects, and did not find a correlation. Raitakari *et al.*³⁰², in their study of post-prandial endothelial function, noticed a significant change with VOP, but not with FMD ($p < 0.001; n = 12$). Studies incorporating FMD and LDI also fail to demonstrate a significant correlation between these vascular beds^{328;329}. PWA has been widely used to study arterial stiffness, but its use for endothelial function testing is still limited. However, comparisons between PWA and VOP appear to be quite favourable, as confirmed by Lind *et al.*³²⁷, and also by Wilkinson²³².

The association between coronary and peripheral endothelial function is critical. The fact that known risk factors for coronary artery disease can also cause endothelial dysfunction in peripheral vascular beds suggests that non-invasive peripheral methods may allow accurate identification of those at risk of CAD. Clearly, early identification of coronary endothelial dysfunction and risk factor modification, could have profound implications for the health of large sections of society. Such a tool would have to be non-invasive, as well as convenient and relatively cheap, and FMD has been mooted as a possible option. Coronary and brachial arteries are both conduit arteries, and so it is reasonable to assume that FMD, rather than VOP or LDI, most accurately reflects coronary endothelial function. In any case, VOP would probably have to be considered too invasive for this purpose. Findings by Anderson *et al.*³³⁰ demonstrate that coronary endothelial function, when stimulated by acetylcholine, is weakly (but significantly) associated with brachial artery FMD. This association is stronger when reactive hyperaemia is used as the coronary stimulus³³¹.

The widespread acceptance that endothelial damage is a necessary pre-requisite of atherosclerotic disease has generated a surge of interest in *in-vivo* assessment of endothelial function. This review identifies nine methods that are currently being used for assessing endothelial function. VOP, FMD, LDI and Quantitative Coronary Angiography are the most widely used; the other methods, although promising, are still at relatively early stages of development. Each one studies a different vascular bed, and varies in their degree of invasiveness, and there is a lack of consensus regarding method and protocol selection. The perceived “gold standard” for assessing *peripheral* vascular function is VOP^{203;216}, however this perception may well be flawed. What one can say is that VOP is

the “gold standard” for assessing forearm resistance vessel endothelial function, FMD is the “gold standard” for assessing conduit artery endothelial function, and so on. These issues need clarification, preferably with large scale comparative studies.

Chapter Four

Methodology

4.1 Introduction

In this thesis SSc vascular function was studied using several different methodologies. Endothelial function in medium-sized muscular arteries and in the microcirculation, was studied using the established techniques of brachial artery flow-mediated dilatation (FMD) and laser Doppler iontophoresis (LDI). Arterial elasticity of the brachial and carotid arteries, was studied by applying recorded arterial pressure and diameter to established biomechanical algorithms. Viscoelasticity of the carotid artery was studied by plotting measured intra-luminal pressure against diameter to create hysteresis loops.

Accurate measurement of arterial diameter is critical for assessment of both FMD and arterial elasticity/viscoelasticity. This was achieved by use of a high-resolution duplex ultrasound coupled with an echo-locked edge-detection software system, albeit applied in very different ways for each modality. LDI, on the other hand, required the use of an entirely different equipment set-up, as well as a different approach to measurement. This chapter will commence with a discussion on the diagnostic and selection criteria used for SSc subjects. Subsequently, there will be a detailed analysis of the various methodologies used, as well as background discussion on the theory behind them.

4.2 Classification and Diagnosis of SSc

4.2a. American College of Rheumatology Criteria

Systemic sclerosis shares several key clinical features, including skin thickening and Raynaud's phenomenon, with non-sclerodermatous rheumatological and dermatological conditions. In 1980, the American College of Rheumatology (ACR) published preliminary criteria for classifying a condition as SSc, and they are still used today (Table

4.1)³³². These criteria were determined by a multi-centre comparative analysis of 264 SSc patients, and 410 patients with non-sclerodermatous connective tissue diseases such as systemic lupus erythematosus, polymiositis and dermatomyositis. The criteria have a specificity of 97% and a sensitivity of 98% for SSc with respect to these other connective tissue diseases³³².

These are purely clinical criteria and several authors have urged caution in their use. Problems arise because other conditions within the scleroderma spectrum, such as morphea, and even some purely dermatological conditions, such as eosinophilic fasciitis, may present with characteristic skin hardening, but are not SSc^{22;333}. Such problems are related in large part to the experience of the diagnosing clinician. All the SSc patients who took part in this thesis were diagnosed by a single senior consultant rheumatologist.

Table 4.1 ACR classification criteria for systemic sclerosis. The presence of the major criterion, or at least two of the minor criteria, is sufficient to classify a condition as SSc.

Major criterion	Minor criteria
Skin hardening proximal to metacarpo-phalangeal joints	Sclerodactyly
	Digital pitting scars
	Bibasilar pulmonary fibrosis

4.2b. Distinguishing lcSSc from dcSSc

While the ACR criteria facilitate the classification of SSc versus non-sclerodermatous disorders, a further system is needed to differentiate SSc subgroups. The most widely used criteria are those proposed by LeRoy *et al*¹⁷. They found that 90% of SSc patients can be grouped into one of two subtypes; 60% have limited cutaneous SSc (lcSSc), and

30% diffuse cutaneous SSc (dcSSc)¹⁶. In essence the groups are differentiated according to pattern of skin involvement. Thus those with skin thickening limited to the skin of the face and distal limbs are categorised as lcSSc, while those with skin thickening extending to the proximal limbs and trunk are dcSSc (Table 4.2). In this thesis, all patients who had been categorised as SSc, were then sub-categorised into either lcSSc or dcSSc according to the LeRoy criteria, by the same senior rheumatologist.

Table 4.2 Differentiating lcSSc and dcSSc. *LeRoy criterion¹⁷.

	lcSSc	dcSSc
*Skin involvement	face and distal limbs	proximal limbs and trunk
Time to complications	long, maybe 10 years	short, often less than 5 years
Antibody profile	ACA sensitivity 57% ACA specificity 92%	ATA sensitivity 40% ATA specificity 83%
Internal organs	oesophagus, lungs	heart, lungs, kidneys, gut

4.2c. Other tests useful in diagnosis

Serum autoantibody detection can be helpful in reinforcing the clinical diagnosis of SSc. Using immunofluorescence, approximately 96% of patients who fulfil the ACR criteria stain positive for serum anti-nuclear antibodies (ANA)³³⁴. This test is used routinely as a diagnostic aid, however, serum ANA can be detected in most autoimmune connective tissue disorders, so it lacks specificity. Other antibodies are anti-DNA topoisomerase-1

(ATA) and anti-centromere antibody (ACA). These are more specific than ANA but less sensitive, and can be useful in differentiating lcSSc from dcSSc (Table 4.2)³³⁵⁻³³⁷.

Nailfold capillaroscopy is a simple and widely used tool that can help to differentiate SSc from other connective tissue disorders. Features commonly associated with SSc are reduced number of capillaries, avascularity, megacapillaries and microhaemorrhages. The *scleroderma capillary pattern* is characterized by a decreased capillary density and the presence of megacapillaries³³⁸⁻³⁴¹. Reports vary, but Nagy *et al.* in a series of 102 SSc patients, found that 88% of dcSSc patients and 62% of lcSSc patients, displayed the scleroderma capillary pattern³⁴².

While both antibody profiling and nailfold capillaroscopy are useful in a clinical setting, for the purposes of this thesis it was deemed that neither of these tests were sufficiently robust in terms of sensitivity and specificity for use in patient selection. Indeed, their use would inevitably introduce additional sources of error, as well as open up avenues of further investigation which would be outwith the scope of this thesis. Instead, we followed the methodology established by previous investigators by relying solely on the ACR and LeRoy criteria for patient diagnosis and classification, in our case as applied by a single senior rheumatologist whose clinical practice is devoted to SSc.

4.3 Brachial Artery Flow-Mediated Dilatation

4.3a. Background

Flow-mediated dilatation (FMD) is a non-invasive technique for the assessment of *in-vivo* brachial artery endothelial function, the underlying physiological principles of which were discussed in Chapter Three. There were several important reasons for the choice of

FMD for endothelial function assessment. First, the methodology established by Celermajer *et al.*¹⁹⁸ and refined by Corretti *et al.* (and the *International Brachial Artery Reactivity Task Force*)²¹⁸ has been validated in numerous studies and, notwithstanding, is an accepted means of assessing *in-vivo* endothelial function. Second, the aforementioned difficulties relate in part to errors in diameter measurement, however, we were able to minimise this by utilising a novel and highly accurate software system that automatically tracks real-time arterial diameter over a designated number of cardiac cycles (Wall Track System, Pie Medical Systems Software, Maastricht, Netherlands). Third, unlike the other popular method venous occlusion plethysmography (VOP), FMD is entirely non-invasive. Finally, although at least 2 previous studies have used FMD in SSc patients^{343;344}, our study would be the first to provide a meaningful comparison of *in-vivo* endothelial function in the major subtypes lcSSc and dcSSc.

4.3b. Wall Track System

The brachial artery is quite small (typically around 3-6mm) and diameter changes due to FMD are also very small. Traditional B-mode ultrasound is said to provide a resolution of anywhere between 0.15mm and 0.5mm, therefore, a 5% diameter increase in a 5mm wide artery may well be outside the detection range of normal ultrasound³⁴⁵. The Wall Track System (WTS) allows diameter resolutions of between 2 and 8 microns for a pulsatile artery³⁴⁶. Furthermore, WTS has been validated for brachial artery diameter assessment by Hijmering *et al.* In a study of piglet femoral arteries (similar in size to the human brachial artery), they compared the absolute diameter resolution of WTS with intravascular ultrasound (the *gold standard* for arterial diameter measurement), and found

a correlation co-efficient of 0.87³⁴⁷. They also found good intra-session and inter-session reproducibility of brachial artery diameter, as measured *in-vivo* by WTS.

The central piece of equipment in the WTS setup is a duplex ultrasound scanner (Pie 350, Pie Medical Systems, Maastricht, Netherlands) with a 7.5 MHz linear array probe (Figure 4.1). Radio frequency (RF) motion-mode (M-mode) signal output from the scanner is to a standard personal computer (PC) equipped with WTS software. This RF-signal is directly proportional to soft tissue density; so a steady background signal denotes muscle and connective tissue, a high signal denotes the arterial walls, and the vessel lumen registers a negligible signal. WTS recognises two high signals on either side of a low signal as the anterior and posterior arterial walls surrounding the lumen, and continuously monitors the distance between them.

The RF-signal is electrocardiograph (ECG) gated thus allowing WTS to precisely correlate diameter variations with cardiac cycle. Upon obtaining an optimum M-mode image of the brachial artery (Figure 4.2A), WTS is activated and vessel diameter is monitored over 3 to 4 cardiac cycles. WTS first displays the raw RF-signal data (Figure 4.2B). Then the RF signal data over the cardiac cycles is converted graphically to the distension curve over time (Figure 4.2C). The sampling rate of WTS is 200Hz, so each diameter recording generates a large number of data points. Once the recording is complete, these data points are downloaded onto a spreadsheet (Microsoft Excel, Microsoft Corporation, UK) from which further data analyses, including systolic and diastolic diameters are determined.

Figure 4.1 FMD Equipment Setup. A. PC equipped with WTS; B. Duplex scanner; C. 7.5MHz linear array probe locked place in place over brachial artery by armrest/probe holding device; D. Occluding blood pressure cuff.



Figure 4.2A. Demonstration of optimum brachial artery ultrasound image. Arterial walls are highly resolved, and vessel lumen is clear of debris/atheroma. The red line denotes the M-line, the point at which diameter is recorded. Diameter is recorded at this exact point throughout the testing procedure.

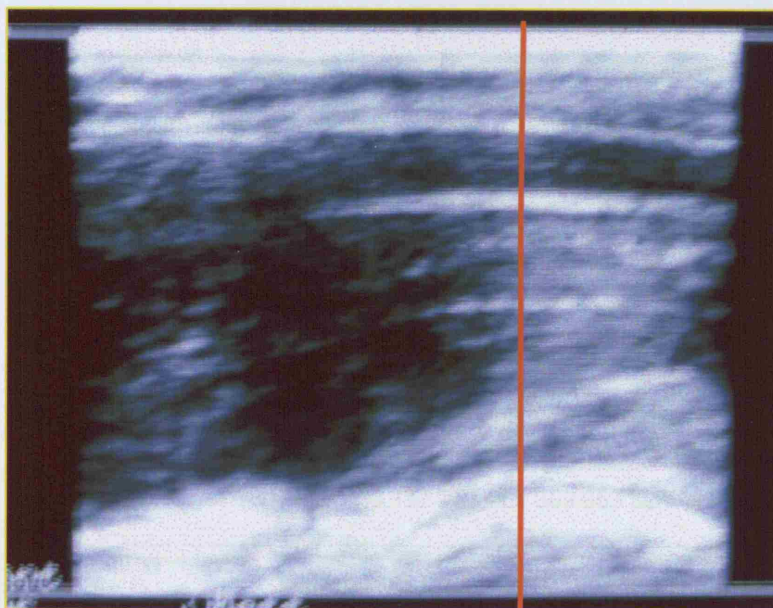


Figure 4.2B. RF-signal output from the brachial artery image above. WTS selects the interface between low and high signals as the intimal/luminal junction, and this is shown by the red markers.

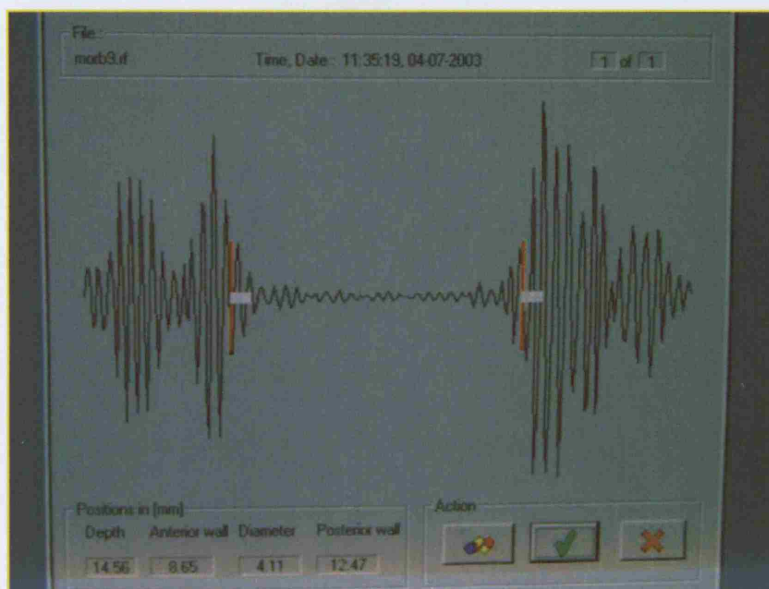
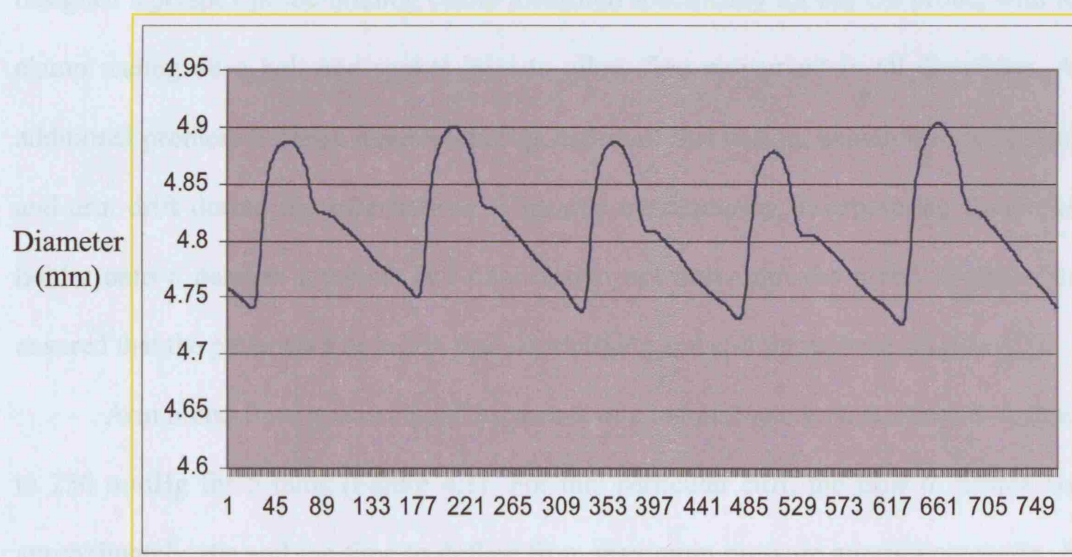


Figure 4.2C. The RF-signal is then plotted against time giving a distension curve over 5 cardiac cycles. The troughs and peaks represent diastolic diameter and systolic diameter respectively.



Data points over 4 seconds (at a sampling rate of 200 Hz)

4.3c. Adjuncts to FMD Assessment

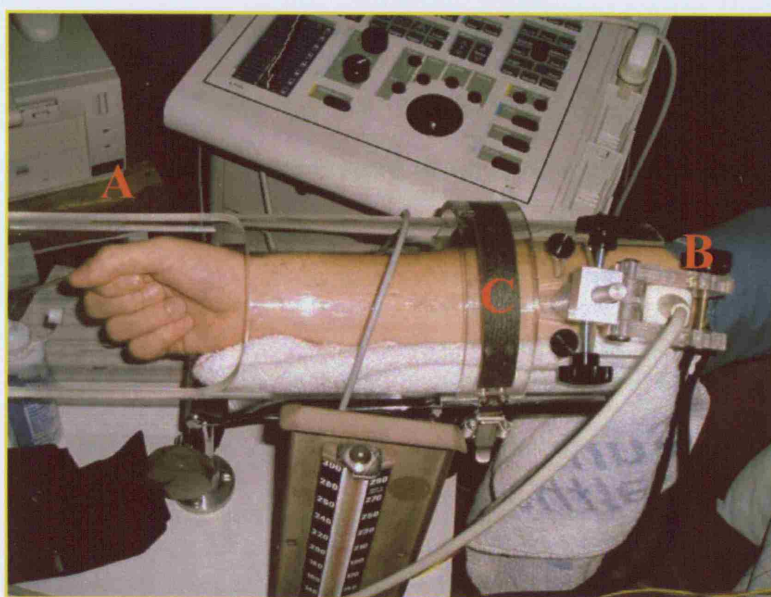
Once an adequate image of the brachial artery is obtained, it is of paramount importance that this precise image is maintained throughout the procedure. Any change in position will result in either a longitudinal or rotational shift in the anterior-posterior line of diameter measurement. The risk of this occurring is greatest during cuff inflation/deflation, and since FMD is the difference between baseline diameter and maximum diameter a shift in position at this time will give an untrue FMD measurement. To minimise this risk Corretti *et al.* have advocated the use of a stereotactic probe holding device, so once an adequate arterial image is acquired the US probe can be locked in place at a fixed point for the duration of the test²¹⁸.

At present there are no commercially available probe holders for FMD, so one had to be designed and built solely for the purposes of our study. The main requirement was that the probe holder should be adjustable for height above skin, be able to rotate in 180°, and be easily locked in place with a one-handed mechanism. To achieve this, I designed a perspex probe-holding clamp measured specifically for our US probe, with the clamp resting on a ball and socket joint to allow free movement in all directions. An additional problem became apparent during testing of this design, namely subject fatigue and arm drift during the examination. This was overcome by incorporating the probe-holder onto a perspex armrest. The final design not only kept the probe in place, but ensured that the patient's whole arm was comfortable and still throughout (Figure 4.3).

Arm blood flow was occluded by means of a manual sphygmomanometer inflated to 250 mmHg for 5 mins (Figure 4.1). For this particular cuff, the time to inflate was approximately 10s and the time to deflate from maximum pressure approximately 5s. As

yet, no study has specifically sought to identify when after cuff deflation maximum FMD occurs, but it is highly likely to occur between 5s and 2 mins from cuff deflation.

Figure 4.3. Perspex armrest and probe holding clamp. A. Perspex armrest; B. Stereotactic probe holding clamp with US probe in place; C. Ball and socket joint on which clamp/probe unit rests.



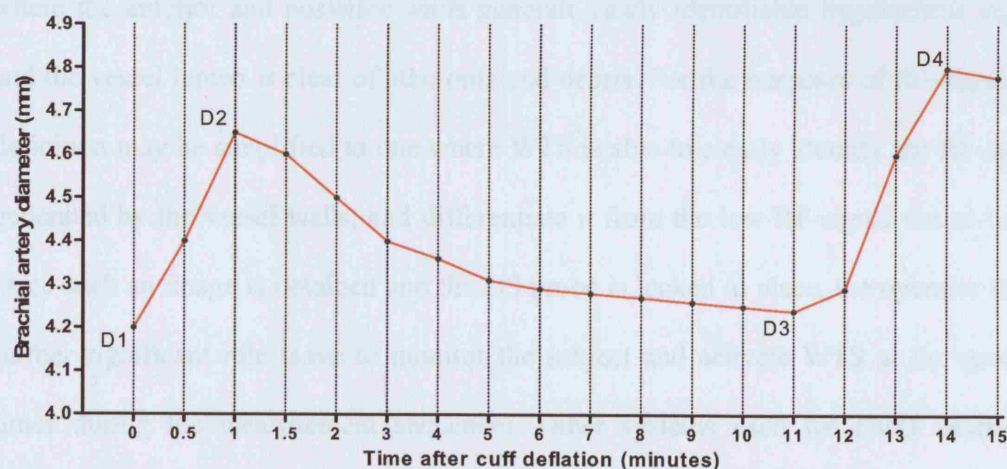
4.3d. Protocol for FMD/GMD Assessment

The FMD/GMD protocol that was used in this thesis is outlined in Chapter five, but a few important background points will be discussed in this section. Our protocol closely follows the guidelines established by the *International Brachial Artery Reactivity Task Force*²¹⁸. The testing procedure begins with an initial baseline brachial artery diameter measurement. Arm blood flow is then arrested with the sphygmomanometer inflated to 250 mmHg for 5 mins. Upon cuff release arterial diameter is measured at 30s, 60s, 90s and 120s, during which time the maximum FMD is recorded; and then every minute for a

further 8 mins, during which time the resumed baseline diameter is recorded. At this point (10 mins after cuff deflation) glyceryl trinitrate (GTN) 500 μ g is given sublingually. Serial measurements are then made every minute for 5 minutes, and the maximum diameter again recorded.

The reason behind making diameter measurements every 30s for the first 2 minutes was that this provided the best chance of measuring maximum FMD, which almost always occurs within 2 mins of cuff deflation^{198;218;290}. Measurements were made every minute thereafter to record the gradual decline in FMD and determine the resumed baseline diameter. Upon GTN administration arterial dilatation occurs more gradually, so one measurement per minute is sufficient to capture the maximum diameter. Normal physiological variations in diameter that occur, for example, in response to breathing were not recorded because we judged that to do so would have made the recording sequence overly complicated, and in any case these variations are known to be very small. A typical measurement sequence using this protocol is shown in Figure 4.4.

Figure 4.4 Diameter measurement sequence. D1. Baseline diameter; D2. Maximum FMD; D3. Resumed baseline diameter; D4. Maximum GTN-induced diameter.



4.3e. Operator and Variability Issues

Obtaining an adequate ultrasound image of the brachial artery from which accurate diameter measurements can be made is notoriously difficult^{198;218;290}. Not only is it a small vessel, it is surrounded by dense connective tissue structures such as the biceps tendon and elbow joint capsule. It is therefore important for anyone undertaking this procedure to undergo an appropriate period of supervised training²¹⁸. All the brachial artery scans performed in the course of this thesis were performed by myself. Prior to commencing formal examinations on patients, I underwent a period of supervised training by an experienced vascular technologist. During this time I performed a total of 100 practice scans (20 volunteers were specifically recruited for this and 5 serial brachial artery scans were performed in each individual). These practice scans were not recorded, but with hindsight, it is arguable that they should have been recorded and improvements quantified. Nevertheless, my training falls within the guidelines advocated by the aforementioned Brachial Artery Task Force. Inter-observer comparisons in Chapters Five and Seven were conducted with my trainer acting as second observer.

In vascular ultrasound generally, a good arterial image may be defined as one where the anterior and posterior walls generate easily identifiable hyperechoic signals, and the vessel lumen is clear of atheroma and debris. For the purposes of this thesis, the definition may be simplified to one where WTS is able to clearly identify the RF-signals generated by the vessel walls, and differentiate it from the low RF-signal vessel lumen. Once such an image is obtained and the US probe is locked in place, the operator has no further significant role (save to monitor the subject and activate WTS at the specified times during the measurement sequence). Other systems used for FMD assessment

generally require one operator to acquire/maintain an adequate image which is recorded on video; and a second operator (using either manual or computer assisted techniques) to review the tape, adjust for diameter changes related to the cardiac cycle, and record FMD and GTN-induced dilatation^{271;348;349}. Such systems are inherently more prone to error, although this has not been quantified²¹⁸. The advantage of WTS is that it substantially reduces the role of the operator, thereby minimising this potential source of error.

Nevertheless, as with all ultrasound procedures the issue of observer variability should be addressed. Variability assessment in this thesis is guided by Hijmering *et al.* who have validated WTS for FMD assesment³⁴⁷, and found good reproducibility of baseline arterial diameter measurements. However, in line with other studies, they found relatively poor reproducibility of post-stimulus brachial artery diameter measurements. Poor same-subject reproducibility of the FMD response is a common problem, and is probably due to the wide range of variables that can affect vasoreactivity. While this limits the use of FMD as a diagnostic/prognostic tool for individuals, it is still very useful for comparing endothelial responses at group level³⁵⁰⁻³⁵². Variability data for baseline brachial artery diameter measurement in this study are shown in Chapter Five.

4.4 Laser Doppler Iontophoresis

4.4a Background

Laser Doppler iontophoresis (LDI) is a non-invasive method that allows *in-vivo* assessment of microvascular endothelial function. The underlying physiological principles are discussed in Chapter Three. There were several important reasons for the choice of LDI as a second tool for endothelial function assessment. First, while FMD

provides data on endothelial function in medium-sized muscular arteries (specifically the brachial artery), LDI allows assessment of microvascular endothelial function, and this is arguably the more important site of vascular injury in SSc. Second, while not as widely used as FMD, the technique is nevertheless growing in popularity and is now a well recognised tool for endothelial function testing, particularly where microvascular injury is suspected. The increasingly widespread application of LDI has led to the development of more refined current delivery protocols, thus minimising the potential problem of current-induced vasodilatation. It has also become apparent that while within subject reproducibility is similar to FMD (in other words relatively poor), like FMD the technique is useful for group comparisons²³⁰. Third, the procedure is non-invasive and well tolerated by all subjects, and this was important for ethical and recruitment considerations. Additionally, it is relatively easy to assemble the necessary equipment for accurate LDI measurement, and the procedure requires minimal operator training.

4.4b Theoretical considerations

When light is reflected off a moving object the frequency of the light wave is shifted, and the amount of shift reflects the speed of the object. This principle is known as the *Doppler effect*, and it underpins the use of LDI for blood flow measurement^{353,354}. For example, laser light of a wavelength of 780nm back scattered off a particle moving in a watery medium with a speed of 1 mms⁻¹ (the estimated speed of a red blood cell in the superficial skin) has a frequency shift of approximately 3.3KHz³⁵⁵. In LDI, laser light is used to illuminate the superficial skin tissues and this light is reflected back by both static cells (skin) and dynamic cells (moving blood). The frequency shifted light reflected back

by the dynamic cells is collected by a photodetector and the resulting photocurrent is processed by a laptop computer to display the *flux*, *concentration* and *speed* of the moving blood. The equations used to compute these variables are as follows³⁵⁵:

$$Flux = \frac{k1 \int_{\omega 2}^{\omega 1} \omega P(\omega) d\omega}{dc^2}$$

$$Conc = \frac{k2 \int_{\omega 2}^{\omega 1} \omega P(\omega) d\omega}{dc^2}$$

$$Speed = k3 \times flux / conc$$

where ω is the frequency of the Doppler shift, $P(\omega)$ is the power of the signal at frequency ω , dc is the intensity of all detected light, $\omega 1$ and $\omega 2$ are the low and high cut off frequencies, and k is a scaling constant. These computations are done automatically by the appropriate computer software and displayed in real-time on the laser perfusion monitor (DRT4, Moor Instruments, Axminster, UK). *Flux* (measured in perfusion units, pu) is the key variable and it reflects the change in blood flow in response to the delivered vasoactive chemical.

The two vasoactive chemicals most commonly used in LDI are acetylcholine (ACh) and sodium nitroprusside (SNP). ACh is a neurotransmitter that triggers endothelium-dependent vasodilatation by providing post-synaptic agonist stimulation of nicotinic ACh receptors of the sensory nerves that regulate neurovascular control of endothelial function^{356;357}. Endothelial cells thus produce edNO which causes vascular smooth muscle cells to relax. SNP on the other hand provides a donor source of nitric oxide direct to vascular smooth muscle cells and so causes endothelium-independent vasodilatation. When in solution these chemicals exist as charged ions which are driven into the skin and onto the microvascular bed when a small current is applied.

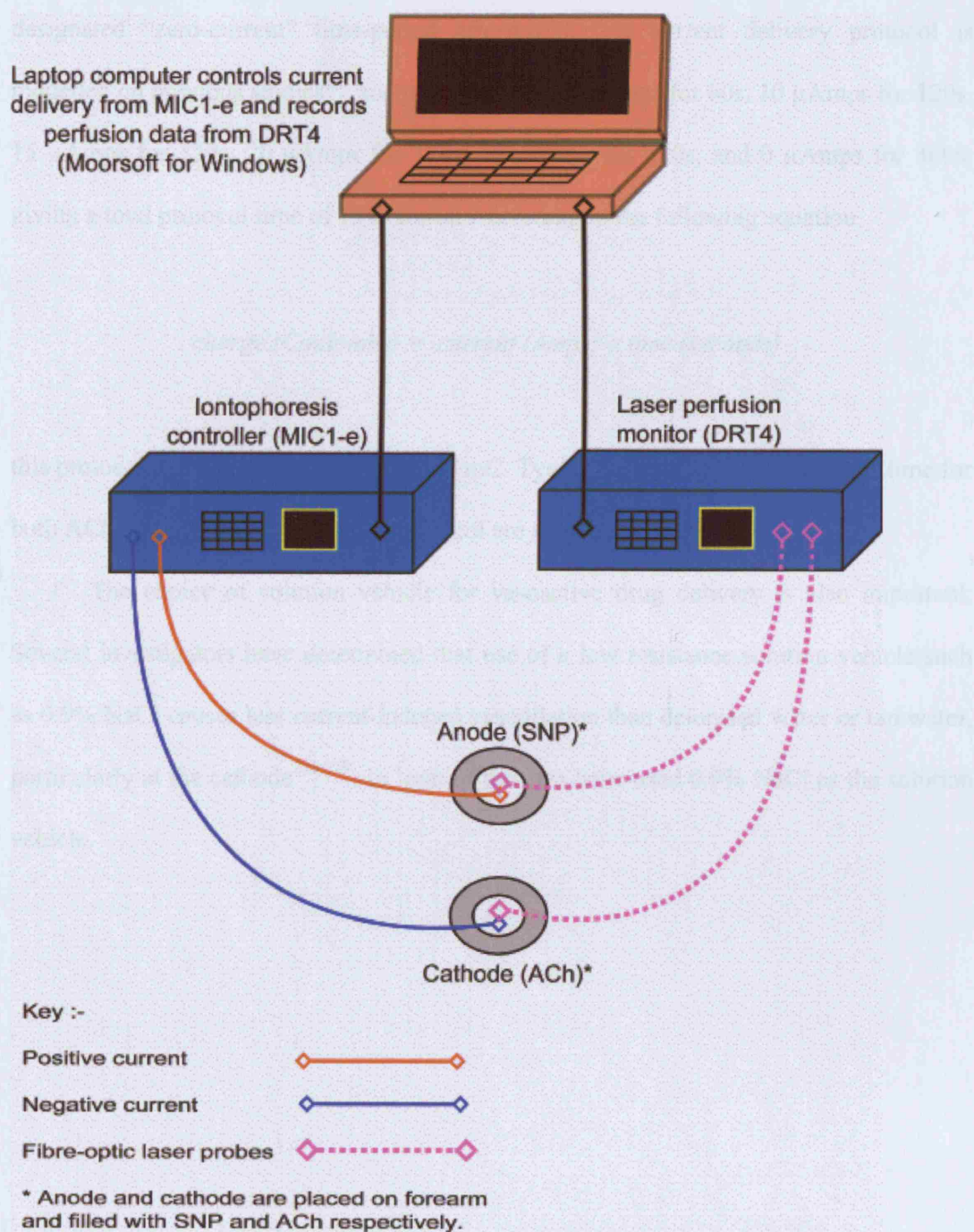
4.4c Equipment setup for LDI

The equipment setup is shown in diagrammatic form in Figure 4.5. A current delivery controller (MIC1-e, Moor Instruments, Axminster, UK) is used to deliver direct current to two perspex iontophoresis electrodes which are secured onto the forearm skin. The electrodes each contain a hollow chamber which is filled with approximately 2ml of the appropriate vasoactive chemical in a solution of 0.9% NaCl. The solutions are made up to ACh 1% for the cathode and SNP 1% for the anode. Current delivery is controlled by a standard laptop computer equipped with appropriate software (Moorsoft for Windows, Moor Instruments, Axminster, UK). Also connected to the electrodes are two fibre-optic laser probes with light source from the DRT4 perfusion monitor. The DRT4 uses solid state laser diodes to provide a laser light source of an approximate wavelength of 780nm. At least two of the probe's glass fibres emit laser light to a single point just under the skin surface electrode at which current (and chemical) are delivered; the rest transmit reflected light back to the monitor. Data from the perfusion monitor is displayed in real-time on the laptop computer and is saved as spreadsheet files (Microsoft Excel, Microsoft, UK).

4.4d Current Delivery Protocol

In order to minimise the effect of current-induced vasodilatation (described in Chapter Three), previous investigators have found the best solution is to deliver the same overall charge (and thus dose of drug) over a longer time-frame. In essence, this means delivering tiny pulses of current over several minutes, as opposed to the previous practice of delivering one large current dose over 30s or 1 minute. The effect on blood flux in

Figure 4.5. Iontophoresis and laser Doppler flux monitoring circuit.



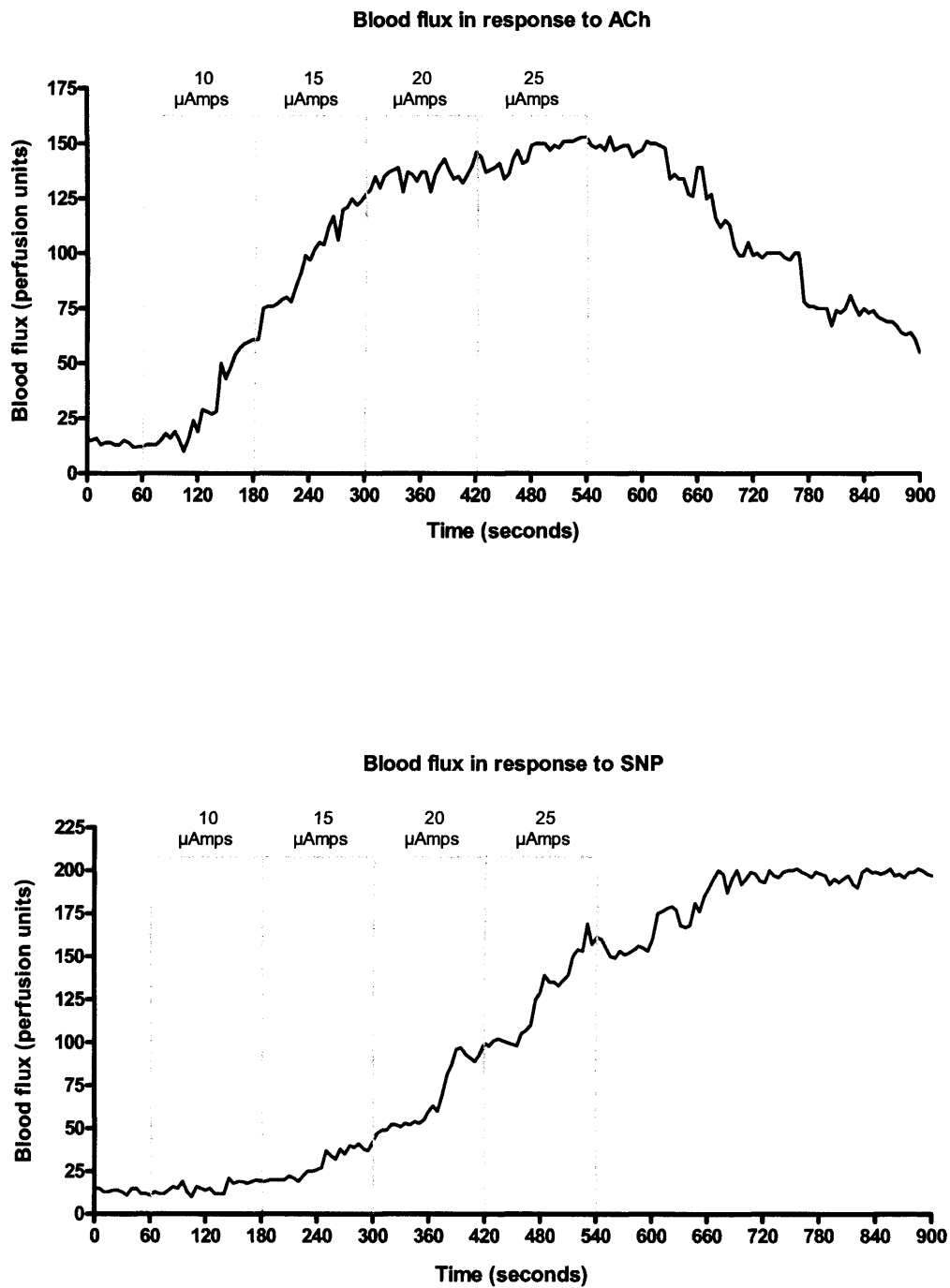
response to the delivered drug is observed throughout the current delivery and for a designated “zero-current” time-period afterwards. Our current delivery protocol is modelled on previous studies²²⁴ and proceeds thus: 0 μ Amps for 60s, 10 μ Amps for 120s, 15 μ Amps for 120s, 20 μ Amps for 120s, 25 μ Amps for 120s, and 0 μ Amps for 360s; giving a total protocol time of 15 minutes. According to the following equation:

$$\text{charge (Coulombs)} = \text{current (Amps)} \times \text{time (seconds)}$$

this protocol delivers a total charge of 8.4 mC. Typical change in blood flux over time for both ACh and SNP when using this protocol are shown in Figure 4.6.

The choice of solution vehicle for vasoactive drug delivery is also important. Several investigators have determined that use of a low resistance solution vehicle such as 0.9% NaCl causes less current-induced vasodilation than deionised water or tap water, particularly at the cathode^{225;229}. In light of this, we have used 0.9% NaCl as the solution vehicle.

Figure 4.6. Typical example of change in blood flux (measured in perfusion units) over time in response to ACh and SNP.



4.5 Arterial Elastic Parameters

4.5a. Background

Assessment of arterial elasticity presents a particular challenge as there is as yet no universally established convention for the characterisation of these parameters, a fact largely due to the highly complex biophysical and biochemical nature of the systemic vascular tree^{358;359}. Over the years, several mathematical constructs have been developed to describe the relationship between arterial pressure and distensibility. These include the central compliance (C), which can be estimated from stroke volume (SV) and pulse pressure from the following equation :

$$C(ml/mmHg) = \frac{SV}{P_s - P_d}$$

The main problem with this parameter is that it fails to take into account wave reflections³⁶⁰. The concept of pulse wave velocity (PWV), defined as the distance a pulse wave travels in a given time period, was introduced by Thomas Young in the nineteenth century, but developed by Bramwell and Hill in 1922³⁶¹. They adapted the Moens-Korteweg equation to physiological variables and established that PWV was inversely related to the square root of distensibility (D). Thus, given a constant arterial pressure, the greater the distensibility of a given arterial segment, the slower the PWV. This concept was described in the context of endothelial function testing in Chapter Three, but in essence it is a function of arterial stiffness, and can be derived from the equation :

$$PWV(m/s) = \frac{d}{t}$$

where d is distance and t is time. This parameter can be used for assessment of “global” arterial stiffness, although one must be careful to consider the effect of reflected waves,

especially when studying large central arteries (such as carotid or femoral, where the propagated reflected wave is likely to be large)²³². For the purposes of this thesis, the main drawback with this approach is the availability and expense of the equipment and software required.

4.5b. Diametric Compliance

Compliance and distensibility are the basic measurements of arterial elasticity. Assuming a linear relationship between these two parameters, they can be defined as the absolute change (compliance) and relative change (distensibility) in volume for a given pressure. The problem is that it is difficult to accurately quantify the *absolute* volume or *change* in volume. Since the artery cannot lengthen during the cardiac cycle, one can assume that changes in cross-sectional area will approximate to change in volume. The compliance co-efficient (CC) and distensibility co-efficient (DC) are both a function of arterial cross-sectional area and arterial pressure, and are given by the following equations :

$$CC(m^2 / mmHg) = \frac{(A_s - A_d)}{(P_s - P_d)}$$

$$DC(mmHg^{-1}) = \frac{(A_s - A_d) / A_d}{(P_s - P_d)}$$

where A_s and A_d are the arterial cross-sectional areas in systole and diastole, and P_s and P_d are the arterial pressures in systole and diastole. If we assume that the artery is circular in cross-section and that the change involved is small compared to the initial diameter, it follows that cross-sectional area change is approximated to by diameter change. This gives us the following equations for compliance (C) and diametric compliance (D)³⁶² :

$$C(m/mmHg) = \frac{(D_s - D_d)}{(P_s - P_d)}$$

$$D(mmHg^{-1}) = \frac{(D_s - D_d)/D_d}{(P_s - P_d)}$$

where D_s and D_d are the internal arterial diameters in systole and diastole, and P_s and P_d are the arterial pressures in systole and diastole. Since the relationship between internal arterial diameter and arterial pressure is non-linear, diametric compliance can only be measured at a pre-specified arterial pressure.

The diametric compliance (D) is the first of three parameters used in this thesis to determine arterial elasticity. The reason for choosing diametric compliance over compliance or distensibility co-efficients is twofold. First, unlike CC and DC , diametric compliance is a function of the relative change in arterial diameter, therefore it allows comparisons of arteries of different calibres to be made (in this case both the carotid and the brachial arteries were studied). Second, the aforementioned Wall Track System was used for all measurements, and this system gives highly accurate diameter readings which makes it very suitable for this parameter.

4.5c. Peterson's Elastic Modulus

This parameter describes the relationship between *stress* and *strain*. Stress is defined as the amount of force exerted per unit area, while strain is a function of stress and is defined as the fractional change in a dimension (volume, area and diameter) that occurs in an artery exposed to a given change in trans-luminal pressure. The stress/strain ratio is also known as the *elastic modulus*. A somewhat simplified version of this highly complex

algorithm was introduced by Peterson in 1960, and is termed *Peterson's elastic modulus* (Ep)³⁶³. Ep can be derived from the following equation :

$$Ep(mmHg) = \frac{(P_s - P_d)}{(D_s - D_d) / D_d}$$

where P is the systolic and diastolic arterial pressure and D is the systolic and diastolic arterial diameter. Like it's inverse, diametric compliance, Ep must be specified at a given arterial pressure, and it is the second parameter that we have used for arterial elasticity assessment.

4.5d. Stiffness index

This is a modification of the Peterson's elastic modulus introduced by Hyashi in 1974. Ep, and indeed diametric compliance are highly dependent upon the arterial pressure. The stiffness index (β) replaces the pulse pressure in the Ep equation ($P_s - P_d$) with the natural log of (P_s / P_d) thus³⁶⁴ :

$$\beta = \frac{\ln(P_s / P_d)}{(D_s - D_d) / D_d}$$

Theoretically, the stiffness index is less pressure dependent than either Ep or diametric compliance and for this reason it was included as the third parameter used for assessment of arterial elasticity.

4.5e. Assessment of Elasticity Parameters

Arterial elastic parameters were assessed in the common carotid artery and in the brachial artery. Assessment requires an accurate means of measuring arterial diameter in both systole and diastole. This was achieved by using the previously described high resolution

duplex ultrasound system with ECG-gated signal output to Wall Track System software (Pie Medical Systems, Netherlands). Systolic and diastolic diameters are then applied to the above equations, along with systolic and diastolic arterial pressure measured using an automated arm sphygmomanometer (Johnson and Johnson, UK). Precise methodology and protocol are described in Chapter Seven.

4.5f. Variability Issues

As with assessment of brachial artery FMD, and indeed any procedure involving operator dependent equipment such as duplex ultrasound, variability issues must be adequately addressed. The methodology used to evaluate vessel distension and elastic indices in this thesis has been previously used and validated by several groups, and all have found errors associated with duplex estimation to be acceptably low³⁶⁵⁻³⁷⁰. Kanters *et al.* have demonstrated inter-observer errors of 3.3% for diastolic diameter, 11.8% for distension and relative distension, 12.3% for the distensibility co-efficient and 19% for stiffness index; their intra-observer errors were slightly lower³⁷⁰. In a similar study, Liang *et al.* have shown a co-efficient of variation of 9.2% for carotid compliance and 10% for the distensibility co-efficient^{365;366}. In the Atherosclerosis Risk In Community (ARIC) study, Arnett *et al.* have shown a high degree of correlation between three different visits (reliability co-efficients were 0.66 for the carotid elastic modulus and 0.77 for the carotid compliance)³⁶⁸. Finally, van den Berkmoortel *et al.* found that reproducibility of the distensibility and compliance co-efficients for carotid artery ranged from 8% to 12%³⁶⁹.

In this study, all analyses were performed by a single, well trained operator (myself). We have calculated intra-session variation of the brachial and carotid artery

systolic and diastolic diameters in 10 individual subjects and with 12 consecutive arterial readings per artery and expressed as a mean co-efficient of variation. Inter-session variation was calculated for brachial and carotid artery systolic and diastolic diameters in 2 individual subjects, each having 12 consecutive arterial readings per session and repeated for 5 sessions on 5 separate days. This was also expressed as a median co-efficient of variation. These data are shown in Chapter Seven.

4.5g. Issues Concerning Blood Pressure Measurement

One particular limitation when assessing these arterial elastic indices, particularly in the common carotid artery, is that the pressure measurements are taken from the brachial artery using a standard automated sphygmomanometer, whereas ideally they should be taken from the arterial segment at which diameters are measured. The reason for this is related to the previously described “augmentation” phenomenon, whereby as the arterial pressure wave travels away from the ascending aorta it encounters reflected waves from the periphery; thus pulse pressure recorded at the brachial artery can be around 18-31% higher than in the carotid artery³⁷¹⁻³⁷³. The advantage of using the brachial artery for assessment of arterial elasticity is that it is possible to get a true reflection of pulse pressure in that arterial segment using a standard automated sphygmomanometer.

However, for carotid artery assessment this presents a potential problem because accurate determination of pressure in this arterial segment would require an invasive procedure – and this would certainly raise difficult ethical obstacles. The solution to this problem lies in the fact that although reflected waves are augmented, this phenomenon is more pronounced in younger, fitter age-groups. And in fact in middle-aged and older

subjects the phenomenon tends to disappear, so the brachial arterial pressure does indeed offer a true reflection of carotid artery pressure^{358;374}. The validity of this approach has been established by several groups³⁷⁵⁻³⁷⁸, and in this thesis the brachial artery pressure has been used for all elasticity calculations.

An additional issue to be aware of with this technique is that pressure is measured non-contemporaneously with diameter. While this is undoubtedly a potential source of error, it is likely to be small, particularly as steps were taken to minimise vasoreactive stimuli which could cause changes in blood pressure. Furthermore, we were careful to maintain a very short time interval between pressure and diameter measurement.

4.6 Arterial Viscoelastic Parameters

4.6a. Background

The algorithms described above provide a static picture of arterial wall biomechanics at a given pressure. While they provide important information regarding vessel elasticity, they are less useful for detailed analysis of how vessel wall properties change during the cardiac cycle (*viscoelasticity*). In order to do this, it is necessary to measure intra-luminal pressure at precisely the site of diameter measurement, and record the change in pressure as the diameter decreases and increases from systole to diastole. Historically, some investigators performed *in-vivo* studies of arterial biomechanics by surgically exposing the vessel under study and recording pressure and diameter data directly^{379;380}. In today's ethically conscious climate, the latter approach could not be justified for study in humans (who are not already due to undergo surgery).

Advances in ultrasound have of-course revolutionised our approach to the study of arterial biomechanics, and echo-locked real-time non-invasive measurement of arterial diameter is now the gold-standard, as described in previous sections. But this still leaves the issue of recording intra-luminal pressure at the site of diameter measurement. In 1989, Kelly *et al.* demonstrated that the palpating pressure waveform in superficial arteries can be measured with high-fidelity tonometry pressure transducers (otherwise known as *applanation tonometry*)³⁸¹. These devices are identical to those described in Chapter Three for assessment of pulse wave velocity.

The arterial wall consists of an arrangement of layers, known as the tunica intima, tunica media and tunica adventitia. As outlined in Chapter Two, each one of these layers can be affected to a greater or lesser extent in SSc. The complexity of the wall ensures that the relationship between pressure and diameter is non-linear. When the diameter change of an arterial segment is plotted against the pressure change in that segment, over a single cardiac cycle, the resulting curve is known as a *hysteresis loop* (Figure 4.7, below). This loop provides a *dynamic* representation of the elasticity of the vessel wall, and there are several algorithms that facilitate mathematical expression of this property.

4.6b. Hysteresis elimination

In 1995, Armentano *et al.* used echo-locked ultrasound in combination with applanation tonometry, in their study of carotid artery wall properties in hypertensives versus control³⁸². They used a complex mathematical analysis which they called *hysteresis elimination*, starting with the following formula:

$$P_{elastic} = P - \eta \cdot \frac{dD}{dt}$$

Their value of η (co-efficient of viscosity) was increased by iteration to obtain the reduction in the hysteresis loop area that maintained the clockwise course of the loop. The purely elastic relationship obtained by their hysteresis elimination procedure was fitted by a logarithmic model which they had previously applied to the description of the elastic properties of large arteries, and transformed into a diameter-pressure curve according to the following formula :

$$D = \alpha + \beta \cdot \ln P$$

Where the diameter (D) is expressed as a function of P, with the constants α and β determined by the fitting procedure. From these calculations they were able to derive diameter-pressure, compliance-pressure, and distensibility-pressure curves over a range of arterial pressures from 50 to 150 mmHg.

Although their work was pioneering, their mathematical approach is unwieldy and not easy to follow, particularly for the relative novice. Furthermore, their approach is difficult to apply to groups with SSc, who manifest a wide range of elastic changes and are therefore likely to have marked differences in their hysteresis loops.

4.6c. Energy dissipation ratio

More recently, Shau *et al.* conducted an *in-vitro* validation of hysteresis methodology³⁸³. They defined the *energy dissipation ratio* (EDR) as denoting the ratio of the energy dissipated to the total energy imparted by blood pressure in systolic phase. In practical terms EDR is given by the following equation:

$$EDR = \frac{A1}{A1 + A2 + A3}$$

where A_1 is the area within the loop (mostly related to the energy dissipated due to the viscoelasticity of the vessel wall), while $A_2 + A_3$ is the area between the x -axis and the unloading side of the loop. EDR is expressed as a percentage, and the higher the viscoelasticity of the vessel wall, the higher the percentage.

4.6d. Dynamic elastic modulus and phase angle

Other algorithms that can be used are the *dynamic elastic modulus* (E), and the *phase angle*^{384;385}. The viscous component of the viscoelastic properties of an arterial wall is a component of E , and is the retarding force, derived from the imaginary part of incremental elastic modulus as:

$$\eta\omega = E \sin \varphi$$

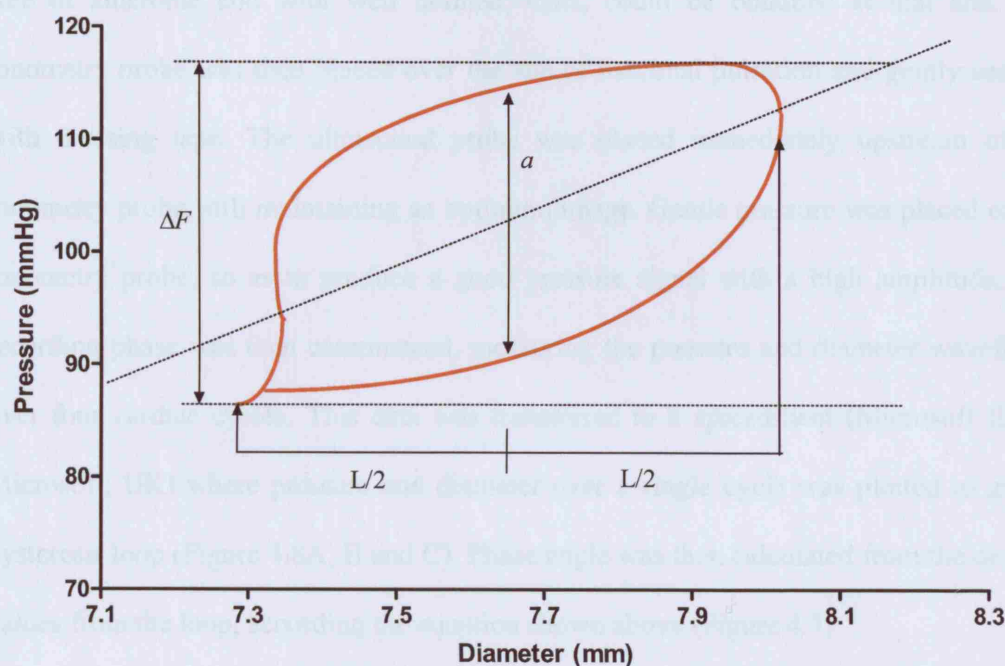
where η is the co-efficient of viscosity and ω is the angular velocity and φ is the phase angle. The phase angle is dependent on the pressure/strain relationship. As the pressure within an arterial segment begins to increase, the radius of the vessel begins to change. However, the change in radius (strain) lags behind the pressure change.

This lag is described by the phase angle, and it is a true reflection of the viscoelastic properties of the vessel wall. It is given by the following equation:

$$\sin \varphi = \frac{a}{\Delta F}$$

Where φ is the phase angle, a is the height between ascending and descending limbs at mid-cycle, and ΔF is the total excursion. In this thesis, phase angle was used for assessment of viscoelastic properties, and these values were derived from the hysteresis loop as shown in Figure 4.7.

Figure 4.7. Diagrammatic representation of calibrated intra-luminal pressure (mmHg) plotted against diameter (mm), otherwise known as a *hysteresis loop*. ΔF = total excursion; a = mid-cycle height; L = loop length



4.6e. Measuring diameter and pressure

All measurements were performed on the common carotid artery. This vessel was chosen because of its ease of access and relatively superficial location. Diameter measurements were performed using the previously described high resolution duplex ultrasound scanner (Pie 350, Pie Medical Systems) with signal output to an echo-locked edge detection software system (Wall Track System, Pie Medical Systems). Arterial pressure was measured by applanation tonometry, *contemporaneously* with diameter measurement, using a probe with a small piezoelectric crystal at the tip (Millar Instruments Inc.). When the probe tip is applied to a pulsating vessel, intra-luminal pressure is translated into a waveform which is recorded on a personal computer.

In practice, this involved palpating the vessel to feel for the site of optimum pulsation. An initial ultrasound scan was performed to ensure that a good arterial image, free of atheroma and with well defined walls, could be obtained at that site. The tonometry probe was then placed over the site of maximal pulsation and gently secured with dressing tape. The ultrasound probe was placed immediately upstream of the tonometry probe, still maintaining an optimum image. Gentle pressure was placed on the tonometry probe, so as to produce a good pressure signal with a high amplitude. The recording phase was then commenced, measuring the pressure and diameter waveforms over four cardiac cycles. This data was transferred to a spreadsheet (Microsoft Excel, Microsoft, UK) where pressure and diameter over a single cycle was plotted to give a hysteresis loop (Figure 4.8A, B and C). Phase angle was then calculated from the derived values from the loop, according the equation shown above (Figure 4.7).

4.6f. Issues concerning pressure measurement

For pressure measurement, the tonometry probe is placed over the point of maximal pulsation and gently pressed down on the artery against underlying rigid structures. Applying pressure on the artery in this way flattens (or “applanates”) the curved surface of the artery, thereby balancing the circumferential stress on the vessel wall, and ensuring that the pressure measured by the sensor is identical to intra-luminal pressure. The amount of pressure one applies is important, because if it is excessive it will distort the biomechanical properties of the artery. We considered that optimum applanation of the vessel was achieved when a reproducible pressure wave with a maximum pulse pressure amplitude was obtained, an approach validated by Armentano *et al*³⁸².

Figure 4.8A. Change in uncalibrated intra-luminal pressure (arbitrary units, AU), during cardiac cycle.

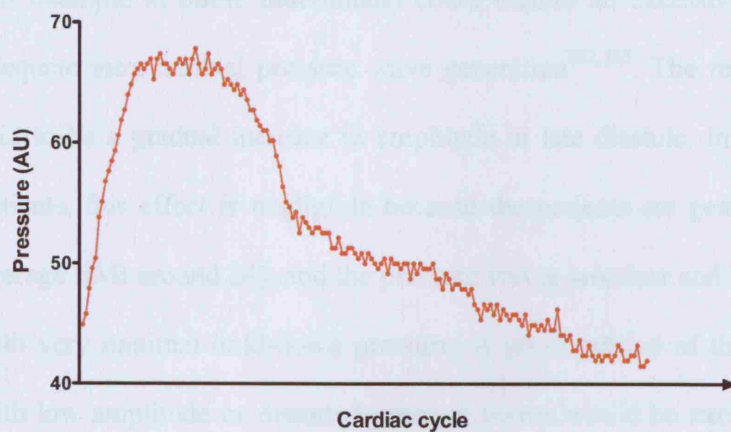


Figure 4.8B. Change in diameter (mm) during cardiac cycle.

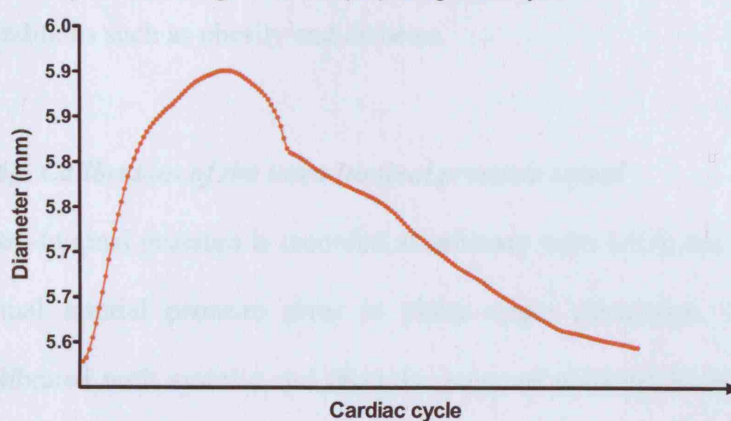
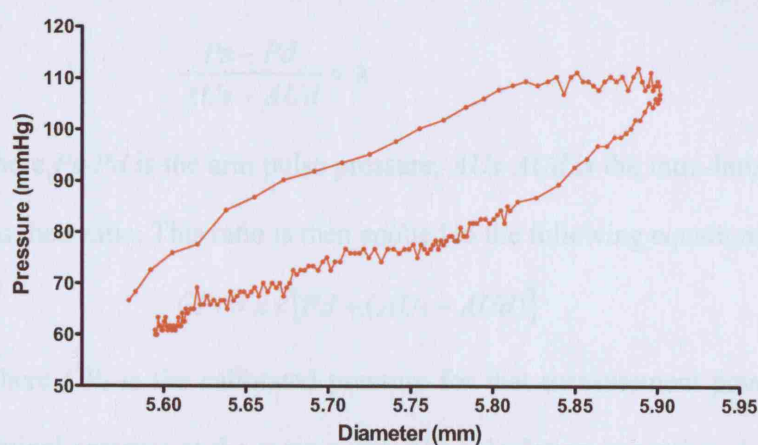


Figure 4.8C. Pressure and diameter plotted against each other to create a hysteresis loop. Pressure has been calibrated, and is expressed as mmHg.



It has been reported in previous studies that excessive tissue overlying the artery (for example in obese individuals) could require an excessive hold-down pressure for adequate intra-luminal pressure wave generation^{382;383}. The resulting wave distortion is said to be a gradual increase in amplitude in late diastole. In our experience with SSc patients, this effect is negligible because the patients are generally quite thin (with an average BMI around 24), and the pressure waves are clear and have good amplitude even with very minimal hold-down pressure. A pre-condition of the study was that subjects with low amplitude or distorted pressure waves would be excluded, but in practice this did not happen. However, clearly this effect should be considered when studying other conditions such as obesity and diabetes.

4.6g. Calibration of the intra-luminal pressure signal

Intra-luminal pressure is recorded as arbitrary units (AU), but it must be calibrated with actual arterial pressure prior to phase angle calculation. The pressure signal was calibrated with systolic and diastolic pressure obtained from a single measurement of brachial arterial pressure using a standard automated arm sphygmomanometer. The calibration procedure was performed according to the following equations:

$$\frac{Ps - Pd}{AUs - AUd} = x$$

where $Ps-Pd$ is the arm pulse pressure, $AUs-AUd$ is the intra-luminal pulse pressure, and x is their ratio. This ratio is then applied to the following equation:

$$CP_1 = x \times [Pd + (AU_1 - AUd)]$$

Where CP_1 is the calibrated pressure for that measurement point and AU_1 is the intra-luminal pressure at the same point. The calculation is continued for CP_2 (with AU_2) and

so on, throughout all measurement points of the cardiac cycle. This procedure was performed on a spreadsheet (Microsoft Excel, Microsoft, UK).

4.6h. Variability issues

Intra-session variability for systolic and diastolic diameters and intra-luminal uncalibrated pressures, was calculated in 10 individual subjects and with 12 consecutive arterial readings per subject. Inter-session variability for systolic and diastolic diameters and intra-luminal uncalibrated pressures was calculated in 2 individual subjects, each having 12 consecutive arterial readings per session and repeated for 5 sessions on 5 separate days. Results were expressed as mean co-efficients of variation and are displayed in Chapter Eight. Ideally, inter-observer variability should also have been assessed but was not done so. The reason for this was because the technique used for generating the pressure signal required much practice, and only one individual (myself) was able to devote sufficient time to perfect the technique. Unfortunately, the timeframe that we had did not permit another individual to become sufficiently trained for fair comparisons to be made.

Chapter Five

An Assessment Of Brachial Artery

Endothelial Function In SSc

Subgroups vs. Healthy Control

5.1 Introduction

Systemic sclerosis is a rare multi-system disorder of unknown aetiology that is characterised by hardening of the skin and connective tissues³⁸⁶. The condition is now considered to comprise a group of disorders collectively known as the “scleroderma spectrum”, and these are classified according to clinical features such as disease duration and extent of skin involvement^{386;387}. Most patients (90%) belong to either the lcSSc subset, or the dcSSc subset¹⁶. Although these subsets differ in terms of symptomatology, disease progression and severity, both are characterised by widespread vascular involvement, which forms the basis of major complications such as digital ischaemia, renal crisis and pulmonary hypertension^{78;387}.

The pathophysiology of vascular disease in scleroderma is still poorly understood, however, it is thought that endothelial cell dysfunction plays a major role. Vascular endothelial cells form a thin layer on the intimal surface of the arterial wall, and they are responsible for the release of a variety of vasoactive mediators which work together to maintain normal vessel tone and coagulation status. Dysregulated release of the vasodilator edNO could certainly be one possible explanation for the heightened vasoconstrictive potential seen almost universally in SSc patients, and *in-vitro* studies have indeed demonstrated reduced edNO mRNA expression in SSc subjects. But that is only part of the story. Disordered endothelial cell function may also affect release of the potent endothelium-derived vasoconstrictor endothelin-1 (ET-1), and several studies have demonstrated higher circulating levels of this hormone in SSc patients.

While there is a broad consensus that endothelial dysfunction is an important event in SSc pathogenesis, the evidence thus far comes from *in-vitro* studies. The aim of

this study therefore, is to conduct a comparative *in-vivo* analysis of endothelial function in lcSSc, dcSSc, and healthy control subjects, using the technique of brachial artery flow-mediated dilatation (FMD). Brachial artery smooth muscle function was also assessed using glyceryl trinitrate (GTN mediated dilatation - GMD). This method is well established and provides a reliable indication of endothelial function/dysfunction at group level. Furthermore, we have adapted the established protocol to incorporate a computer software system (Wall Track System) that allows highly accurate real-time arterial diameter measurements.

5.2 Patients and Methods

The study was approved by the local hospital ethics committee according to the principles of the Declaration of Helsinki. Sixty subjects were studied in total, 20 with lcSSc, 20 with dcSSc, and 20 healthy age-matched and sex-matched healthy controls. Systemic sclerosis was defined according to the criteria of the American College of Rheumatology^{332;388} and was diagnosed by a single senior rheumatologist. All recruited SSc patients had had their condition for at least 2 years. Patients with severe lung disease requiring intra-venous vasodilator or ambulatory oxygen therapy were excluded. Controls were selected from volunteers working in or associated with the Departments of Surgery and Rheumatology. For each subject we recorded age, gender, height and weight (for BMI calculation), and TVRS score. A sample of venous blood was taken for serum cholesterol.

Our protocol for FMD and GMD measurement closely follows the guidelines laid out by the International Brachial Artery Reactivity Task Force^{198;290}. In order to minimise the effect of potentially vasoactive parameters, all subjects were asked to avoid alcohol,

caffeine and nicotine on the study day. Measurements were performed in a quiet temperature-controlled room, with the subject seated comfortably in a 45° reclining posture, such that they were able to remain entirely still for the whole procedure. After a 10 minute acclimatisation period, right arm blood pressure and pulse were recorded using an automated pressure monitor (Dynamap Compact TS, Johnson & Johnson Medical, Newport, UK). The right forearm was placed in a specially-designed perspex armrest with a probe holding clamp at the level of the antecubital fossa, and a standard manual sphygmomanometer cuff was placed around the upper arm (Figure 1a). Using a duplex vascular ultrasound scanner (Pie 350, Pie Medical Systems, Maastricht, Netherlands) with a 7.5 MHz linear array probe, the brachial artery was identified at a consistent location medial to the biceps tendon. Upon acquiring a satisfactory image, with well defined arterial walls and clear of any atheroma (Figure 1b), the probe was locked in place so as to maintain a consistent image throughout the procedure. After a baseline diameter measurement, the blood pressure cuff was inflated to 250 mmHg for a period of 5 minutes. The cuff was then sharply deflated, and brachial artery diameter was measured at 30 second intervals for 2 minutes and then at 1 minute intervals for a further 8 minutes. Ten minutes after cuff deflation, a final measurement was made to ensure the diameter had returned to the original baseline. GTN was then given as a 500 µg sublingual tablet and diameter measurements were taken every minute for a further 5 minutes. At the end of the session, a venous blood sample was taken to assess serum lipid levels.

All studies were performed by a single trained individual, using the same duplex scanner (Pie 350, Pie Medical Systems, Maastricht, Netherlands) with signal output to a high-resolution, echo-locked, ECG-gated, wall tracking system (Wall Track System, Pie

Medical Systems, Maastricht, Netherlands). This system has been validated for the assessment of brachial artery FMD³⁴⁷, and has been described in detail in Chapter Four.

5.3 Data analysis and statistical methods

As previously stated, dilatation due to post-cuff flow increase was labelled FMD. Dilatation in response to sublingual GTN was labelled GTN-Mediated Dilatation (GMD). Each recording returned arterial diameter over at least 3 cardiac cycles. The average systolic and diastolic diameter over the first 3 cardiac cycles in each recording was used for calculation of FMD and GMD indices. We chose to record over 3 cycles for two reasons, first because to record over longer time intervals would have required larger gaps between recording points and could have resulted in missing the maximum diameter, and second we were concerned that to record over longer timeframes would add to subject fatigue and potentially introduce errors owing to minute drifts in arm position. Diameters were designated as follow: pre-FMD baseline systolic diameter = $Ds1$ and pre-FMD baseline diastolic diameter = $Dd1$; post-FMD peak systolic diameter = $Ds2$ and post-FMD peak diastolic diameter = $Dd2$; pre-GMD baseline systolic diameter = $Ds3$ and pre-GMD baseline diastolic diameter = $Dd3$; and post-GMD peak systolic diameter = $Ds4$ and post-GMD peak diastolic diameter = $Dd4$. FMD was calculated as $Ds2 - Ds1$ for systolic phase and $Dd2 - Dd1$ for diastolic phase. GMD was calculated as $Ds4 - Ds3$ for systolic phase and $Dd4 - Dd3$ for diastolic phase. Group comparisons were made using the percentage increase in diameter from baseline to peak (designated FMD% and GMD%) using the following equations:

$$1. \text{ Systolic FMD}\% = \frac{FMD}{Ds1} \times 100 = \frac{Ds2 - Ds1}{Ds1} \times 100$$

$$2. \text{ Diastolic FMD}\% = \frac{FMD}{Dd1} \times 100 = \frac{Dd2 - Dd1}{Dd1} \times 100$$

$$3. \text{ Systolic GMD}\% = \frac{GMD}{Ds3} \times 100 = \frac{Ds4 - Ds3}{Ds3} \times 100$$

$$4. \text{ Diastolic GMD}\% = \frac{GMD}{Dd3} \times 100 = \frac{Dd4 - Dd3}{Dd3} \times 100$$

Basic patient data were compared using one-way ANOVA, with the exception of gender for which a Chi-square test was used. Mean pre-FMD and pre-GMD diameters across groups were compared using a paired T-test. Group means for both systolic and diastolic phases were compared using one-way ANOVA, and where significant differences were found a general linear model (GLM univariate) was used to adjust for the potentially confounding effects of age, systolic blood pressure, heart rate, serum cholesterol, BMI and TVRS score. *P* values of 0.05 or less were judged statistically significant.

All studies were performed by a single trained operator (myself) whose chief role was to obtain a satisfactory image of the brachial artery and lock the probe in place at that point. All subsequent diameter measurements were performed by WTS, with the role of the operator reduced to inflating/deflating the cuff and ensuring the subject remained comfortable and still. Intra-observer variability for acquisition of baseline brachial artery diameter was calculated by assessing intra-session variability and inter-session variability. For intra-session baseline brachial artery diameter, this was done by recording

arterial diameter 4 separate times (giving a total of 12 readings) in the same recording session in 10 individuals, and expressed as a mean co-efficient of variation. Inter-session variability was calculated from 12 readings of baseline brachial artery diameter in 2 individuals on 5 different days, and expressed as a mean co-efficient of variation. Although all study subjects were studied by a single observer, potential inter-observer differences were nevertheless assessed in a further 5 subjects in whom after the main observer had acquired the baseline brachial artery diameter and removed the ultrasound probe, a second observer (the vascular technologist responsible for my training) was called in to the room to acquire a fresh baseline brachial artery diameter reading. Inter-observer variability was also expressed as a mean co-efficient of variation. All variability data were calculated using healthy volunteers (drawn from the control group). All statistical analyses were performed using SPSS for Windows version 11.

5.4 Results

Characteristics of patients and controls are described in Table 5.1. There were no significant differences between groups for age, gender, BMI, serum lipids or TVRS score. However, both systolic blood pressure ($p < 0.001$) and heart rate ($p = 0.013$) were progressively and significantly higher between groups from control to lcSSc to dcSSc.

All diameter measurements are in millimetres. All diameter results are displayed in Table 5.2. There was no significant difference between mean pre-FMD baseline brachial artery diameter and mean pre-GMD baseline brachial artery diameter for either systolic [4.07(Ds1) and 4.12 (Ds3), $p = 0.71$] or diastolic phases [3.95 (Dd1) and 3.99 (Dd3), $p = 0.75$]. Diameter changes pre/post-FMD and pre/post-GMD for systolic and

diastolic phases are illustrated as before-after plots in Figures 5.1 and 5.2. Differences in FMD% and GMD% between groups for both systolic and diastolic stages are illustrated as vertical scatterplots in Figure 5.3 and Figure 5.4.

Group comparisons of mean FMD% and mean GMD% are summarised in Table 5.3. FMD% in both systolic and diastolic phases was significantly lower in the dcSSc subgroup compared to both lcSSc and control ($p<0.001$), and after adjustment for potentially confounding variables this finding was still significant ($p=0.003$). There was no significant difference between groups for GMD% in either systolic ($p=0.626$) or diastolic phases ($p=0.773$).

Intra-session variability was 2.4%, and inter-session variability was 3.3%. Inter-observer variability was 5.9%, and intra-session variability for the second observer (assessed in 5 subjects with a total of 12 baseline diameter readings per subject and expressed as a mean co-efficient of variation) was 2.7%. Inter-session variability was not assessed for the second observer.

Table 5.1. Subject Data. Values are means (SD).

	Healthy controls	lcSSc	dcSSc	<i>p</i> value
Number	20	20	20	
Sex (M/F)	6/14	4/16	4/16	<i>p</i> = 0.96
Age	51.6 (12.8)	50.4 (13.2)	53.6 (12.1)	<i>p</i> = 0.72
Systolic blood pressure (mmHg)	113.2 (12.9)	127.0 (14.4)	134.8 (13.0)	<i>p</i> < 0.001
Diastolic blood pressure (mmHg)	73.8 (9.4)	71.3 (8.7)	70.6 (7.7)	<i>p</i> = 0.47
Heart rate	69.9 (9.9)	76.9 (14.5)	81.7 (11.5)	<i>p</i> = 0.013
BMI	24.3 (3.0)	25.9 (6.6)	25.4 (3.5)	<i>p</i> = 0.53
Serum cholesterol	4.52 (0.9)	5.02 (0.9)	5.1 (1.4)	<i>p</i> = 0.18
TVRS score	0.35 (0.7)	0.65 (0.8)	0.85 (0.8)	<i>p</i> = 0.13

Table 5.2. Results of all brachial artery diameter measurements across groups. Ds1 = systolic pre-FMD diameter, Dd1 = diastolic pre-FMD diameter; Ds2 = systolic post-FMD diameter, Dd2 = diastolic post-FMD diameter; Ds3 = Systolic pre-GMD diameter, Dd3 = diastolic pre-GMD diameter, Ds4 = systolic post-GMD diameter, Dd4 = diastolic post-GMD diameter. Values are means (SD).

Systolic Diameter (mm)		Diastolic Diameter (mm)	
Ds1	4.07 (0.74)	Dd1	3.95 (0.73)
Ds2	4.45 (0.79)	Dd2	4.32 (0.78)
Ds3	4.12 (0.74)	Dd3	3.99 (0.73)
Ds4	4.67 (0.67)	Dd4	4.58 (0.71)

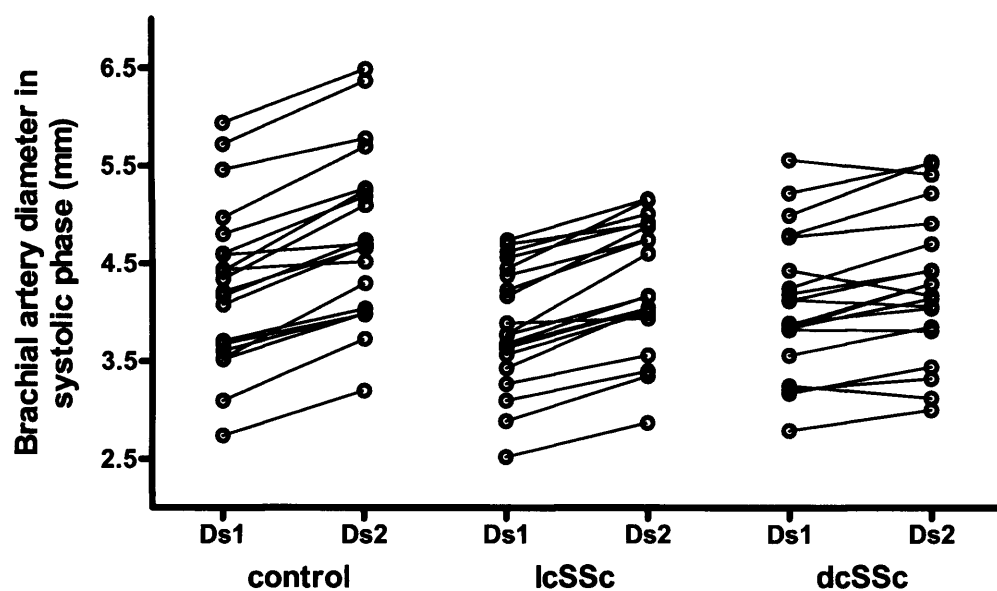
Table 5.3. FMD% and GMD% between groups for systolic and diastolic phases. Values are means (SD).

	control	lcSSc	dcSSc	p value
Systolic phase				
FMD%	12.1 (5.4)	11.4 (4.8)	4.98 (5.4)	p = 0.003*
GMD%	12.9 (7.0)	13.9 (5.4)	15.2 (9.4)	p = 0.626
Diastolic phase				
FMD%	12.8 (5.8)	11.1 (3.7)	5.22 (5.8)	p = 0.003*
GMD%	16.4 (9.8)	14.5 (5.4)	15.8 (8.9)	p = 0.773

*Adjusted for the potentially confounding variables age, heart rate, systolic blood pressure, BMI, serum cholesterol and TVRS.

Figure 5.1. Before and after plots showing change in brachial artery diameter pre- and post-FMD in systolic phase (A) and diastolic phase (B).

A.



B.

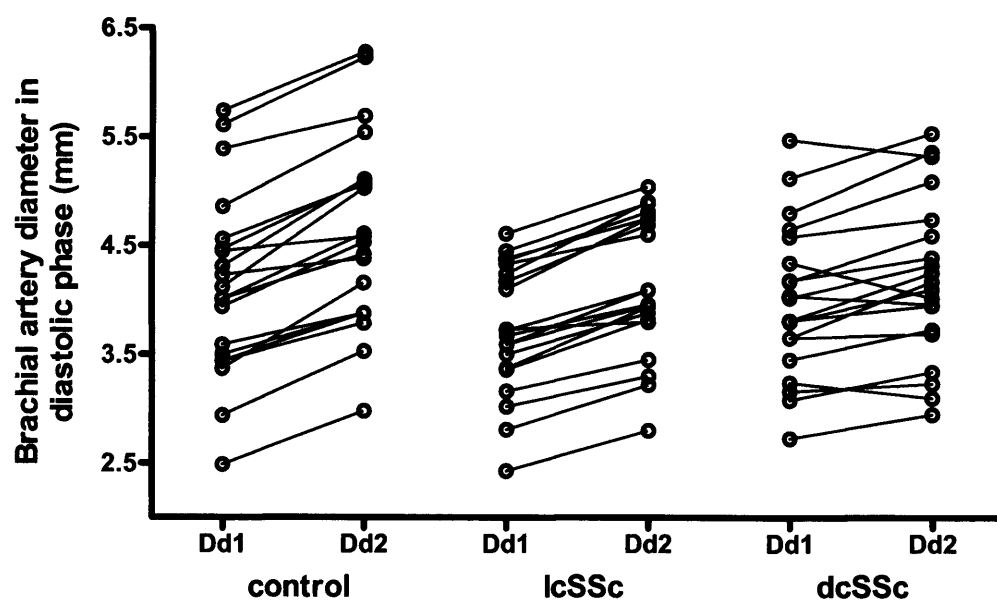
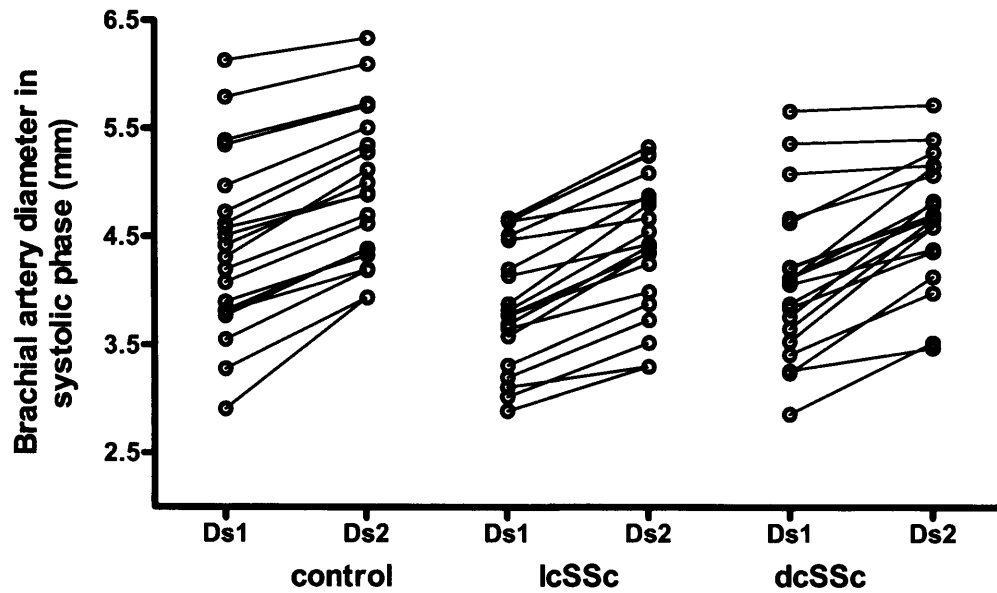


Figure 5.2. Before and after plots showing change in brachial artery diameter pre- and post-GMD in systolic phase (A) and diastolic phase (B).

A.



B.

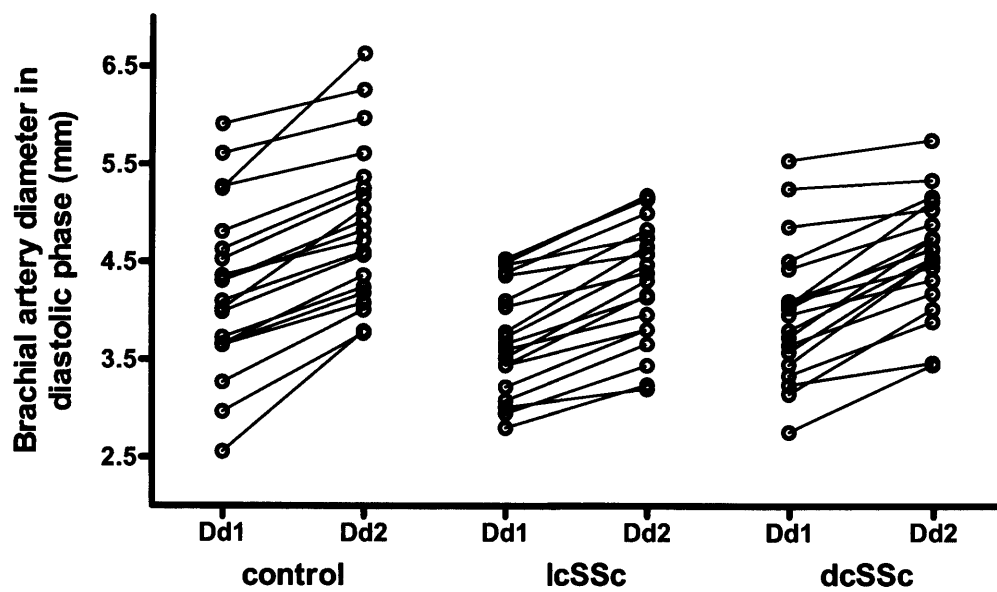
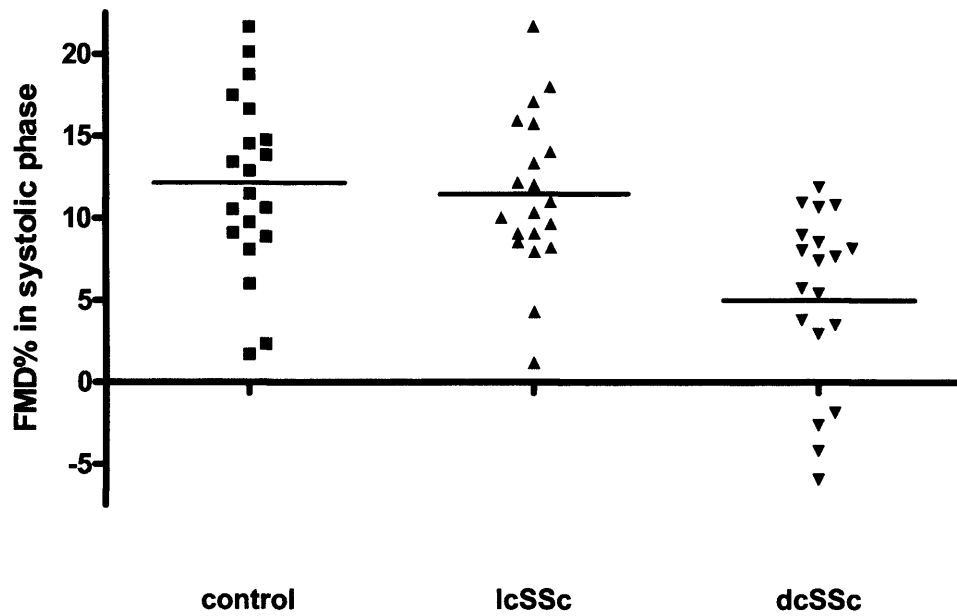


Figure 5.3. Vertical scatterplots showing FMD% in systolic phase (A) and in diastolic phase (B). Horizontal bar represents the mean.

A.



B.

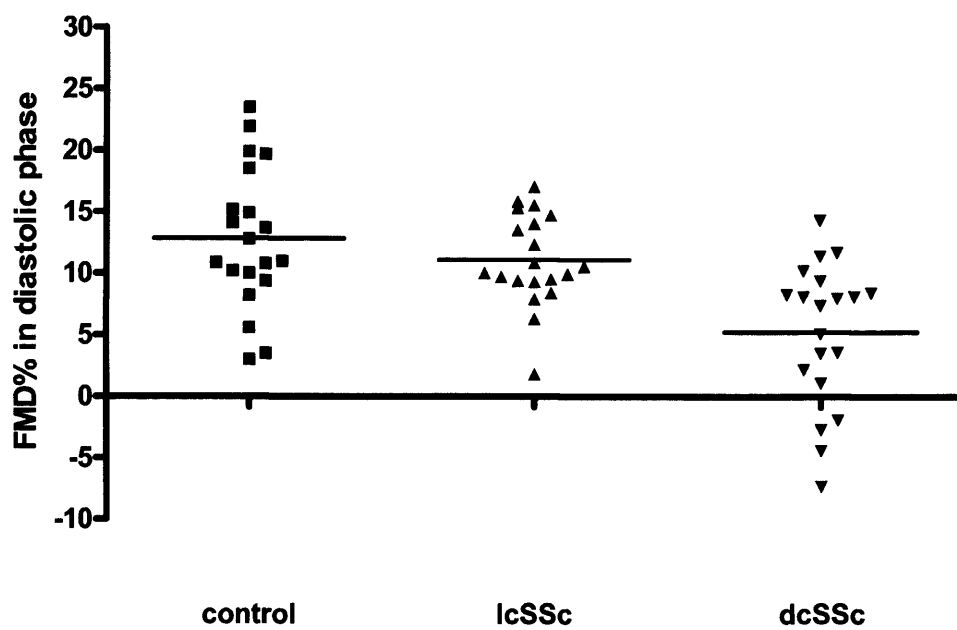
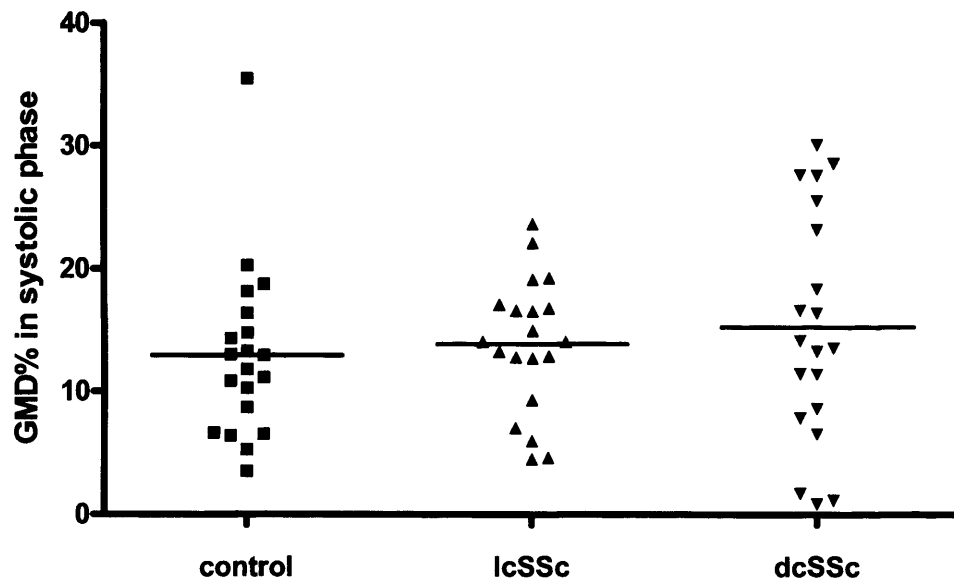
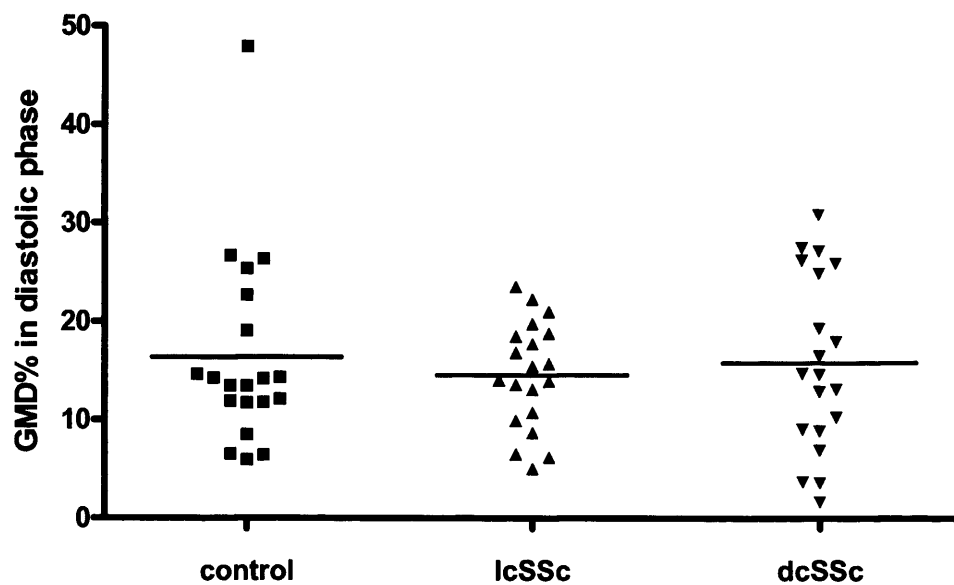


Figure 5.4. Vertical scatterplots showing GMD% in systolic phase (A) and in diastolic phase (B). Horizontal bar represents the mean.

A.



B.



5.5 Discussion

This study sets out to determine *in-vivo* endothelial function in groups of lcSSc and dcSSc subjects, and in healthy controls, using the technique of brachial artery flow-mediated dilatation. This technique was selected after a thorough critical review of all methods currently being employed for *in-vivo* endothelial function assessment (Chapter Three). Before embarking on this study we attempted to adequately address certain problematic issues in relation to FMD assessment, the most important of which is the issue of observer-induced errors.

In the recent past (and even today in some cases), the technique was often performed using relatively low-resolution ultrasonic equipment. The brachial artery would be visualised with a manually held probe, both before and after cuff inflation/deflation, and the recorded ultrasound images would then be analysed at a later time with arterial diameter being measured manually using ultrasound calipers²¹⁸. With this kind of approach there is little wonder that the technique gained a reputation for inaccuracy and error. In this study, every available precaution was taken to minimise the error from these potential sources. All scans were performed by a single individual (myself) who had performed approximately 100 practice scans of the brachial artery prior to commencing the study. High resolution ultrasonic equipment was used, and a stereotactic probe holding device was specifically designed that allowed an accurate arterial image to be maintained at all times. Most importantly, an automated wall-tracking software system (Wall Track System) ensured that all arterial diameter measurements were highly accurate and free of observer bias.

As previously stated, the use of WTS for FMD assessment has been validated in a study by Hijmering *et al.*³⁴⁷, where they compared groups of smokers vs. non-smokers, and they specifically sought to quantify the variability errors associated with the technique. In their study they reported intra-observer variability of 1.1% and 3.6% for intra-session and inter-session respectively, and inter-observer variability of 4.1%. This compares favourably to my results for intra-observer variability (intra-session 2.4%, inter-session 3.3%), and inter-observer variability of 5.9%. Assessment of intra-session variability is important because it informs as to the accuracy of the first brachial artery diameter reading, which in turn indicates the quality of the image that subsequent measurements will be based on. If the first image is of poor quality then the distension curve will be uneven and diameter readings (which are taken as a mean of three cardiac cycles for both systolic and diastolic phases) will also be inaccurate, and this will be reflected as a higher intra-session variability. Once an accurate initial image is acquired then maintaining the image throughout the testing session is not usually a problem, provided the subject is fully aware of the nature of the test and is accordingly compliant.

By contrast inter-session variability informs as to the ability to reacquire a similarly accurate brachial artery image at a subsequent testing session. It could be argued that this information is not relevant because a higher degree of variability would be expected anyway due to the reactive nature of the muscular brachial artery (which is acutely sensitive to vasoreactive stimuli), and that in any case this study does not require repeated measurements of the same subject. While this is certainly true, the calculated inter-session variability nevertheless does provide an important guide as to the conditions in which the test is done. In theory, by avoiding all potentially vasoreactive stimuli prior

to commencing diameter measurement, the brachial artery should return to its resting state, and presumably this diameter is fairly constant. A low inter-session variability thus gives a degree of reassurance that the conditions of the test are such that vasoreactive influences are minimised and the FMD test is performed on an artery in its resting state.

Although all the observations were performed by a single operator, it was important that my ability in acquiring accurate baseline arterial images was compared with another operator, and therefore inter-observer variability was determined in a small group of healthy volunteers ($n = 5$). The inter-observer variability was 5.9%, indicating a reasonable degree of concordance in baseline brachial artery diameter measurement between myself and the second observer, but a certain amount of caution should be exercised when interpreting this result. Firstly, the relatively small sample size for this assessment must be acknowledged before drawing conclusions. Secondly, this stage of the study was conducted after FMD data collection was complete, and therefore my experience at using the armrest to acquire an accurate brachial arterial image was greater than the second observer. Ideally I should have compared my performance to someone of equivalent experience to myself. Despite these caveats the inter-observer statistic for this study does at least give some indication that different observers can acquire similarly accurate baseline brachial arterial images. With hindsight, perhaps inter-observer differences could have been better assessed by involving the second observer at the beginning of the study, and ensuring that both observers followed the same learning curve.

Hijmering *et al.* also assessed the variability of the FMD and GMD response in individuals, and found co-efficients of variation of 13.9% and 9.1% respectively. This is

actually quite a respectable degree of reproducibility for these responses, and is better than has been reported by other groups. There is a broad consensus that FMD and GMD responses have poor within-subject reproducibility, and this is said to be a reflection of the difficulty of maintaining an environment completely free of vasoreactive stimuli, as well as a reflection of the inaccuracies of some arterial diameter measuring systems. Perhaps WTS provides a degree of accuracy that can overcome this reproducibility issue. However, there is an equally broad consensus that FMD and GMD responses have good reproducibility when determining endothelial/smooth muscle function at group level. In this study, within-subject variability for FMD and GMD responses was not assessed because these have been well documented by other groups. Further, the aim of the study was to determine group differences in a cross-section of lcSSc, dcSSc and control subjects. To assess FMD and GMD variability would require a more longitudinal study design, and this would have created unnecessary additional ethical considerations.

Mean FMD% and mean GMD% are the parameters that reflect endothelial function and smooth muscle function respectively, and data was acquired for both systolic and diastolic phases. It is conventional to perform all calculations from the diastolic diameter as this is regarded as the resting diameter, however, WTS allows accurate measurement of both systolic and diastolic diameter and so in this study both these phases were used for FMD% and GMD% determination. Preliminary group comparisons were made using a regression model (one-way ANOVA) because this procedure produces a one-way analysis of variance for a quantitative dependent variable (either FMD% or GMD%) by an independent variable (either healthy control or lcSSc or dcSSc), and allows one to test the hypothesis that several means (in this case 3) are equal.

A general linear model (GLM Univariate) was then used to determine the effect of certain covariates on the quantitative dependent variable. The covariates for this study were variables which were thought likely to effect endothelial function such as BMI, systolic and diastolic BP, age, serum cholesterol and TVRS score. These covariates were applied to all 3 groups (including healthy control) because all subjects were selected based on limited criteria which did not include absence of any particular cardiovascular risk factor or disease.

The *first hypothesis* of this thesis states that endothelial dysfunction can be demonstrated *in-vivo* in SSc patients, and that the dcSSc subset manifests greater endothelial dysfunction in line with it's clinically more aggressive profile. Using our FMD technique we have demonstrated that FMD% (i.e. endothelial function) is significantly impaired in the dcSSc subgroup compared to both lcSSc and control, even after incorporating the effect of the aforementioned covariates ($p = 0.003$). By contrast GMD% (i.e. smooth muscle function) is similar in all three groups ($p = 0.773$). Most investigators agree that endothelial dysfunction is a critical event in SSc pathogenesis, but there is as yet no consensus as to how this process fits into the overall evolution of the disease. There is even less understanding of how the pathological processes might differ between the two major SSc subsets. This study does not attempt to provide answers to these important questions, however, it does provide for the first time a convincing *in-vivo* demonstration that endothelial function is specifically impaired in the dcSSc subgroup compared to both lcSSc and healthy controls.

Chapter Six

A Comparative Analysis of Microvascular Endothelial Function Between SSc Subgroups

6.1 Introduction

Microvascular disease is a prominent feature of SSc and is responsible for common conditions such as Raynaud's phenomenon and digital gangrene²². In the lcSSc subset Raynaud's phenomenon often predates frank SSc by several years, while in dcSSc Raynaud's phenomenon is less frequent and typically occurs concurrent with other features of the disease²². However, patients in both subsets often require hospital admission, either for drug treatment using intravenous vasodilators such as the prostacyclin (PGI₂) analogue *iloprost*⁷⁴, or for debridement and amputation of gangrenous digital ulcers³². As discussed in Chapters Two and Three, the underlying cause of this microvascular disease is thought to involve injury to microvascular endothelial cells, leading to impaired release of vasoactive mediators. Of critical importance is the balance that exists between mediators, such as edNO and ET-1, in maintaining normal vessel tone and coagulation status. Impairment of this balance is said to cause a heightened vasoconstricting potential and an increased clotting tendency, ultimately leading to repeated microvascular occlusions and ischaemia. Evidence that this may be occurring in SSc comes mainly from *in-vitro* studies that have identified dysregulated release of these and other mediators in endothelial cell cultures and serum analyses of affected patients^{44;51;126;155;156;159}.

In Chapter Five, the FMD method was utilised to demonstrate impaired *in-vivo* brachial artery endothelial function in the dcSSc subgroup, compared to lcSSc and healthy controls. Given the clinical presentation of SSc vascular disease, one obvious limitation of the FMD method is that it studies medium-sized muscular arteries, rather than looking at the microvasculature directly. Thus, in order to provide a more complete

picture of endothelial function in SSc, we decided to proceed to an investigation of microvascular endothelial function.

The LDI technique has been applied in at least two previous studies for microvascular endothelial function assessment in SSc^{389,390}. However, while these studies compared SSc versus healthy control, they failed to differentiate between SSc subtypes in their study group. Bearing in mind our findings in Chapter Five, where we found selective endothelial impairment in dcSSc versus lcSSc, and since previous studies have already looked at SSc versus healthy control, we decided to pursue a well-defined goal for this study. Thus the aim was to use the LDI technique to perform a comparative analysis of *in-vivo* microvascular endothelial function in lcSSc versus dcSSc.

6.2 Patients and Methods

The study was approved by the local hospital Ethics committee in accordance with the guidelines of the declaration of Helsinki. Subjects were recruited according to the selection criteria and method described in Chapter Four. Steps were taken to avoid vasoactive parameters as previously described. Arm blood pressure was recorded using an automated sphygmomanometer (Johnson and Johnson, UK), height and weight measurements were made and BMI calculated, and the patient's TVRS score determined. Patients were then seated in a relaxed position with their forearm resting on a flat table-top. At the end of the session a blood sample was taken for measurement of cholesterol (taken at the end to avoid vasoreactive effect from tourniquet use in certain individuals).

By definition, all dcSSc patients had mild to moderate forearm skin thickening, while all lcSSc patients had minimal forearm skin involvement. Iontophoresis

measurements were performed using the methodology outlined in Chapter Four and the equipment setup shown in Figure 4.5. All measurements were performed at a consistent location on the ventral aspect of the right forearm midway between elbow and wrist, at a site free from broken skin lesions and superficial veins. Any surface hairs were shaved and the site was cleaned with deionised water to ensure a neutral pH. The iontophoresis skin surface electrodes were then applied and secured with adhesive tape.

The negative electrode (cathode) was filled with a solution of 1% acetylcholine (ACh; Sigma-Aldrich Company Ltd., Dorset) and the positive electrode (anode) filled with a solution of 1% sodium nitroprusside (SNP; Fluka Industries, Switzerland). ACh stimulates the microvascular endothelial cells to produce nitric oxide and thus causes “endothelium-dependent vasodilatation”, while SNP acts directly on microvascular smooth muscle cells to cause “endothelium-independent vasodilatation”. The phenomenon of *current-induced vasodilatation* has been described in Chapters Three and Four. In order to minimise this effect we selected a protocol that delivers an adequate total charge but spreads delivery of this charge over a longer timeframe, an approach that has been validated in several previous studies^{224;229}. This protocol consisted of fifteen 60s periods as follows (Table 6.1) : period 1 = no current, periods 2 and 3 = 10 μ Amps, periods 4 and 5 = 15 μ Amps, periods 6 and 7 = 20 μ Amps, period 8 and 9 = 25 μ Amps, and periods 10 to 15 = no current. A typical response from this protocol is shown in Figure 4.6 (Chapter Four). After completion of the current delivery protocol, the electrodes were removed and the forearm and the site cleaned with deionised water.

Table 6.1 Current delivery protocol.

period	time (s)	current (μAmps)
1	60	0
2	120	10
3	180	10
4	240	15
5	300	15
6	360	20
7	420	20
8	480	25
9	540	25
10	600	0
11	660	0
12	720	0
13	780	0
14	840	0
15	900	0

6.3 Data analysis and statistical methods

The blood flux at the start of the current protocol was taken as the baseline level, and the highest blood flux either during or after current delivery was taken as the peak. The ratio of peak flux to baseline flux was taken to represent the effect of vasoactive drug on blood flux in that individual, and group comparisons were made using the mean (SD) peak/baseline ratio for each group. Basic patient data were compared using one-way ANOVA, with the exception of gender for which a Chi-square test was used. *P* values of 0.05 or less were judged statistically significant.

Flux data were log transformed prior to analysis. Group means were compared using one-way ANOVA, and where significant differences were found a general linear model (GLM univariate) was used to adjust for the potentially confounding effects of systolic blood pressure, heart rate, serum cholesterol, BMI and TVRS score. Inter-session

variability was determined by calculating within subject variation of right forearm responses to ACh and SNP in two healthy individuals on five separate days. All statistical analyses were performed using SPSS for Windows version 11.

6.4 Results

A total of 30 patients were studied, 15 with a diagnosis of lcSSc and 15 with a diagnosis of dcSSc. Both groups were well matched for age and sex. None of the patients suffered from any serious internal organ complications of SSc at the time of study (determined by absence of symptoms such as shortness of breath and recent normal renal function tests). All patients were positive for anti-nuclear antibody. No patient had intravenous vasodilator treatment (*iloprost*) in the 6 months preceding the study, and no patients were on oral vasodilator treatment at the time of the study. Basic patient data are shown in Table 6.2. Systolic blood pressure was significantly higher in the dcSSc subset ($p = 0.002$), but otherwise there were no significant differences between groups.

Within subject increases in flux from baseline to peak are depicted in Figure 6.1A and B for ACh and Figure 6.2A and B for SNP. Group differences in mean ACh and SNP peak/baseline ratios are illustrated as vertical scatter plots in Figure 6.3A and B. Mean (SD) peak/baseline ratio in response to ACh was 1.79 (0.28) for lcSSc and 1.48 (0.33) for dcSSc. Preliminary analysis suggested that response to ACh was significantly lower in the dcSSc group ($p = 0.009$), however, after adjustment for the cumulative effect of potentially confounding variables (systolic blood pressure, age, heart rate, serum cholesterol level, BMI and TVRS score), this difference was no longer statistically significant ($p = 0.154$). Mean (SD) peak/baseline ratio in response to SNP was 1.63

(0.29) for lcSSc and 1.48 (0.28) for dcSSc, this difference was not statistically significant ($p = 0.167$).

Mean co-efficient of variation for the ACh and SNP response studied in the same (right) arm in two individuals on 5 separate days was 13.7% and 17.4% respectively.

Table 6.2. Basic subject data. Values are means (SD).

	lcSSc	dcSSc	<i>p</i> value
Number	15	15	
Age	50.27 (8.75)	54.3 (8.56)	0.21
Gender (M/F)	5/10	5/10	1
BMI	25.7 (4.41)	25.2 (3.3)	0.71
Systolic blood pressure	124.4 (8.854)	134.47 (7.32)	0.002
Diastolic blood pressure	71.6 (6.104)	71.93 (5.55)	0.88
Heart rate	77.13 (11.04)	80.47 (9.76)	0.39
Serum cholesterol	4.79 (1.07)	4.81 (0.95)	0.96
TVRS	0.67 (0.82)	1 (0.76)	0.26

Figure 6.1A. High and low plot showing change in blood flux from baseline to peak in response to ACh for each individual in the lcSSc group.

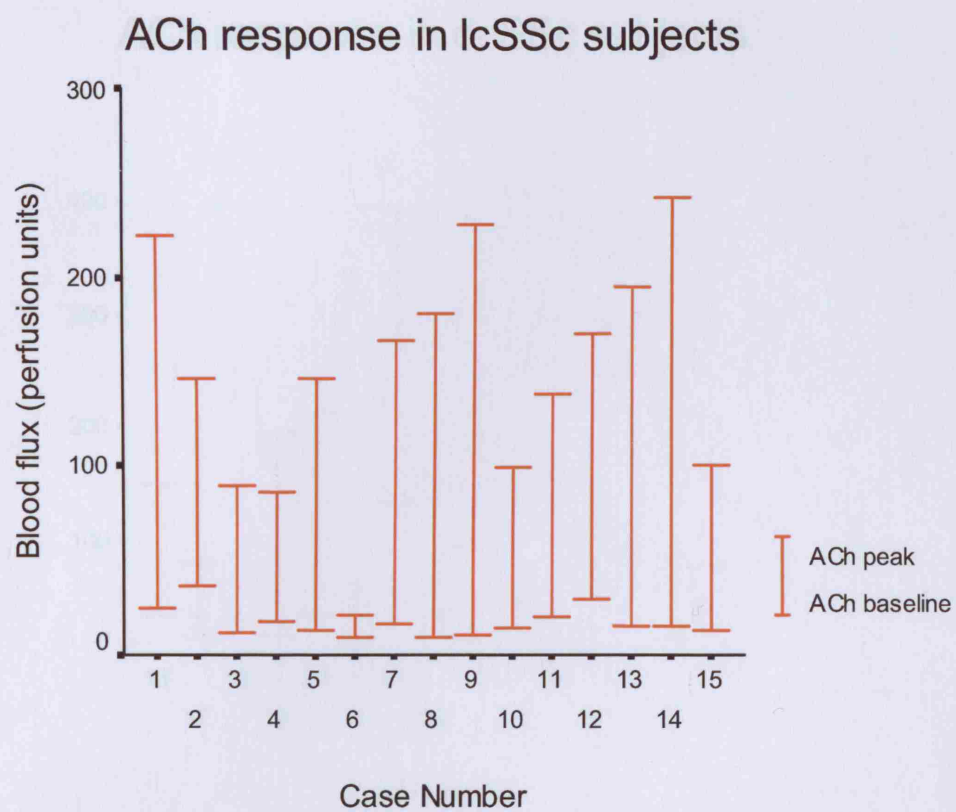


Figure 6.1B. High and low plot showing change in blood flux from baseline to peak in response to ACh for each individual in the dcSSc group.

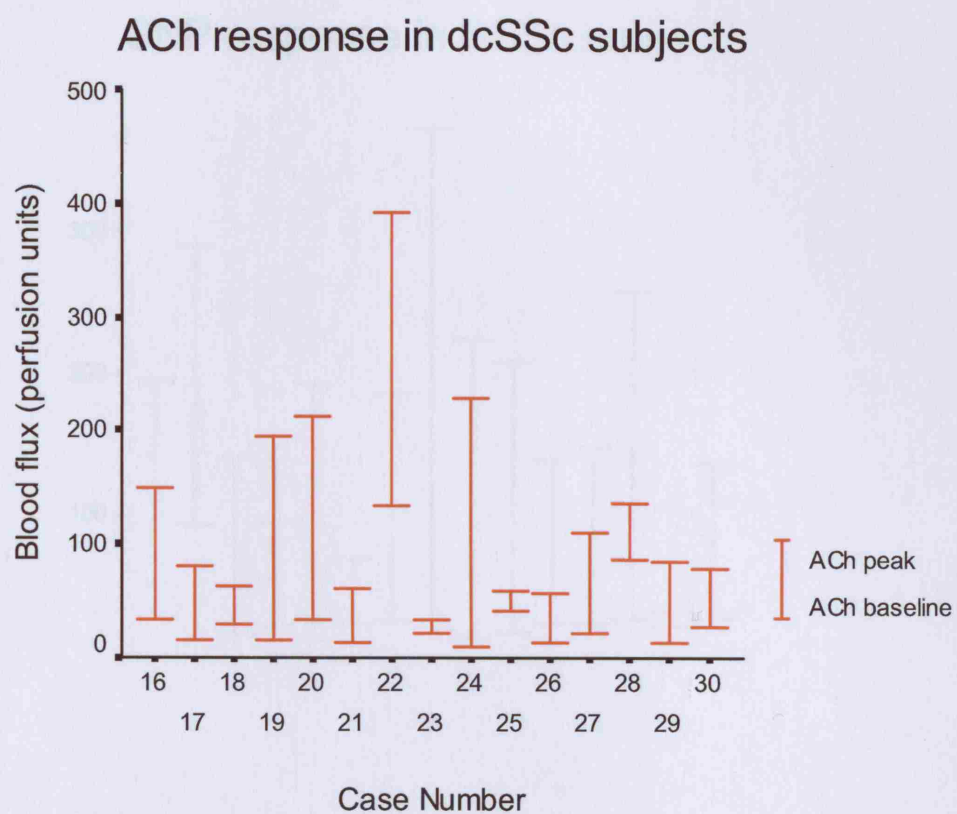


Figure 6.2A. High and low plot showing change in blood flux from baseline to peak in response to SNP for each individual in the lcSSc group.

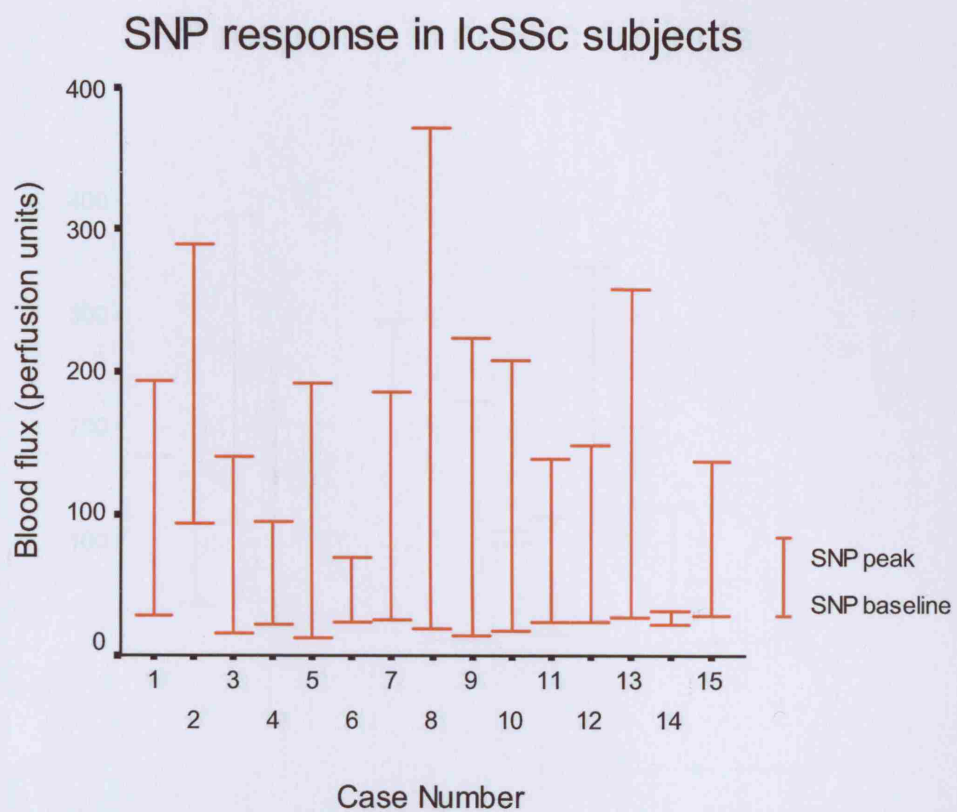


Figure 6.2B. High and low plot showing change in blood flux from baseline to peak in response to SNP for each individual in the dcSSc group.

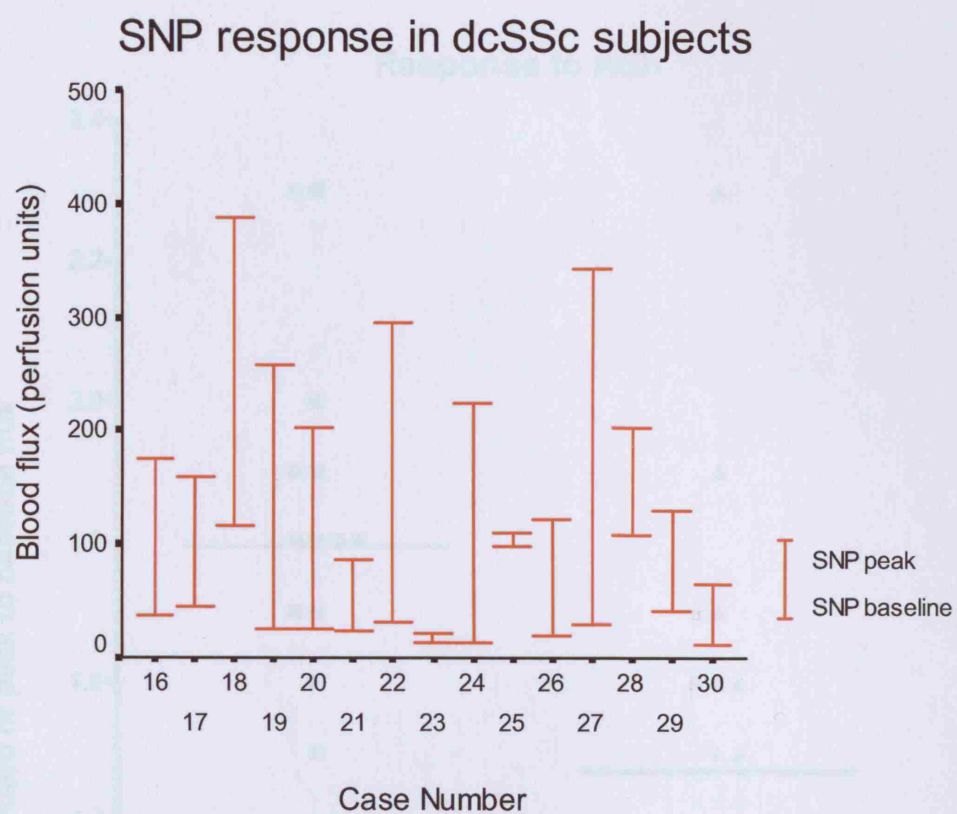
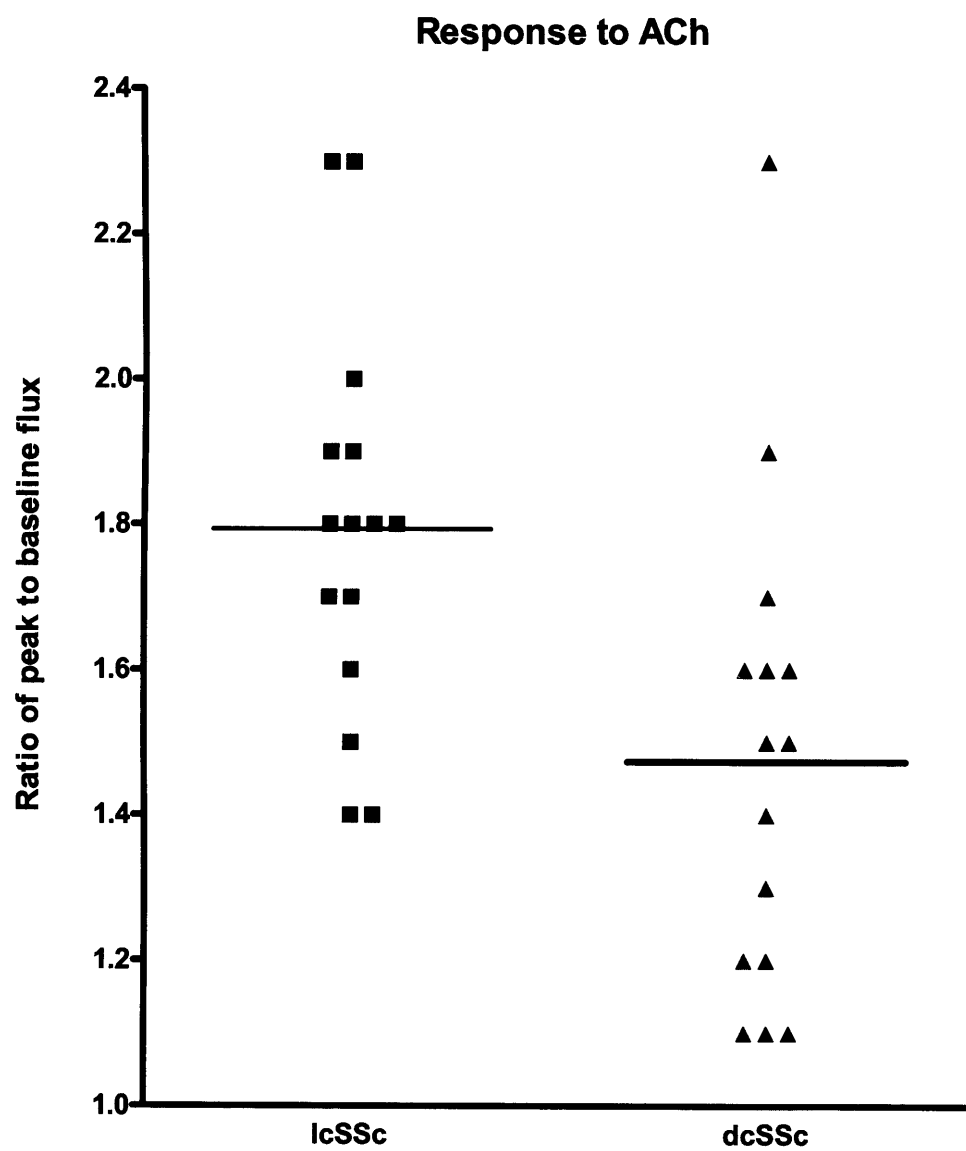
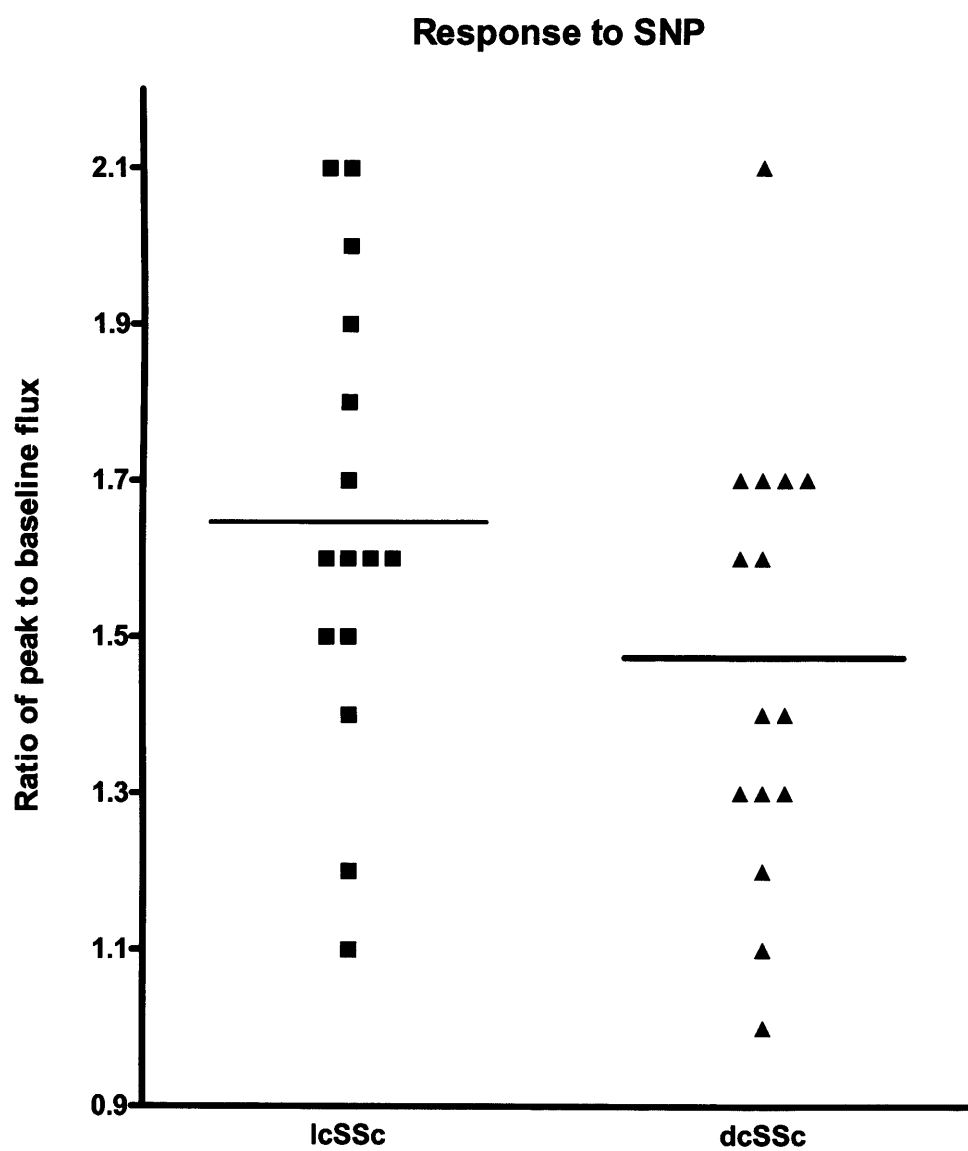


Figure 6.3A. Vertical scatter plot comparing mean ratios of peak/baseline flux in lcSSc and dcSSc in response to ACh (data log transformed prior to analysis).





6.5 Discussion

Laser Doppler iontophoresis is an attractive and increasingly widely used technique. It's advantages are that it is non-invasive, easily tolerated, inexpensive and relatively straightforward to perform. In particular, the LDI technique is relatively immune to criticisms of observer-induced error. Unlike FMD, which relies on the skill of the operator in being able to acquire an initial accurate image of the brachial artery, the LDI technique requires no such operator skill. Instead, the operator merely prepares the skin surface and applies the electrodes, then fills the chambers with the appropriate fluid, and the rest is done automatically by the iontophoresis controller, perfusion monitor and computer software. Provided that the protocol and study site remain constant, in theory the technique should have good reproducibility.

In practice however, LDI suffers from the same problems as other techniques that assess *in-vivo* endothelial function, namely poor reproducibility of the endothelial response in individuals. Kubli *et al.* conducted a study specifically addressing reproducibility issues in LDI, and they reported a mean co-efficient of variation of 10% for ACh responses and between 10% and 20% for SNP responses²³⁰. Their results are better than most other investigators who have reported mean co-efficients of variation of between 10% and 20% with both ACh and SNP^{224;391}. They also found that both ACh and SNP responses vary according to the site of study (for example, left arm versus right arm, or forearm versus digit), and they suggested that strict standardisation of the recording site was necessary to minimise reproducibility errors. In this study we ensured that all measurements were performed at precisely the same site in each subject (namely the volar aspect of the right forearm midway between elbow and wrist). Reproducibility of

this approach was assessed by measuring responses in the right forearm of two healthy individuals each studied on a separate day for 5 days, giving mean co-efficients of variation for ACh and SNP of 13.7% and 17.4% respectively, and this compares favourably with other groups.

As discussed in detail in Chapter Three, the most common pitfall associated with the LDI technique is *current-induced vasodilatation*, the phenomenon whereby the applied current itself causes or contributes to the vasodilatory response. In order to minimise this effect, the approach currently favoured by most practitioners of LDI was adopted, namely the use of a low resistance solution vehicle (0.9% NaCl) and the use of smaller currents spread over a longer timeframe. This latter adaptation to the protocol was introduced by Ramsay *et al.* who found that by spreading delivery of the total charge over a longer time, it is possible to avoid the current-induced effect yet still observe the drug-induced effect²²⁴. One potential criticism of our protocol is that we have not sought to specifically quantify the effect of current-induced vasodilatation on the results for ACh and SNP mediated dilatation. We have instead been guided by previous studies that have reported this effect to be acceptably low when using similar protocols and equipment.

Another factor which might arguably have the potential to affect the result is the skin resistance at the site of measurement. This could be especially relevant in SSc because it is possible that the increased skin thickness in these subjects could adversely affect skin conductance. In a standard electrical circuit resistance can be given by the following equation, known as *Ohm's Law* :

$$R = \frac{V}{I}$$

where R is the resistance of the conductor (measured in Ohms), V is the voltage (volts) and I is the current (Amps). Ohm's law is widely applied in physics, and although iontophoresis essentially involves an electrical circuit whereby the conducting medium is the superficial skin, it is not clear whether the law can be meaningfully applied to organic tissue. Skin resistance is normally governed by factors such as presence of keratin, epithelial cells, sebaceous glands and subcutaneous fat, and each of these media will conduct differently depending on intra-cellular water content, skin sweat, and cellular and tissue density. The LDI technique used in this study involves measurement of blood flow at a single point using a single laser probe, and it is impossible to know the precise nature of the tissues that lie under this point without performing a skin biopsy. If Ohm's law were to be applied in this case, a subgroup of patients would require skin biopsies to determine how the varying presence of skin structures affects the calculated skin resistance. However, if one were to use a laser Doppler imager that records mean blood flow over a pre-designated area of skin (as opposed to single point measurement), then perhaps Ohm's law could be more applicable. Such an imager was used by Andersen *et al.* in a study of primary Raynaud's versus lcSSc; and they found that lcSSc subjects required a higher voltage to drive the same current, implying that lcSSc patients have greater skin resistance³⁸⁹. In this study, skin resistance was not assessed because we made the assumption that over a short time-frame R is a constant, and thus should not be a factor when one is interested in the change in skin *blood flow* in response to vasoactive drug. The essential pre-requisite for this assumption is the use of an iontophoresis controller (MIC1-e) that ensures delivery of a steady current (I), R remains constant and the unrecorded voltage (V) thus varies according to the current.

LDI has been used in the study of SSc by at least two different groups. In 1998, La Civita *et al.* used the technique on 11 SSc subjects versus 16 healthy controls and found no significant difference between groups³⁹⁰. However, they failed to distinguish between SSc subtypes in their study group (which consisted of 5 dcSSc, 4 lcSSc and 2 “intermediate” SSc). In a more recent study, Anderson *et al.* used a laser Doppler imaging technique to study digital microvascular blood flow in 10 lcSSc subjects versus 11 healthy controls. They found that both endothelium-dependent and endothelium-independent responses were significantly impaired in the study group ($p = 0.028$ and $p = 0.004$, respectively)³⁸⁹.

Using the LDI technique, we found that microvascular endothelial function was significantly impaired in dcSSc versus lcSSc (0.009), a finding that echoes the selectively impaired brachial artery endothelial function identified in the previous chapter. Interestingly however, once a general linear model was applied to correct for the potentially confounding effects of heart rate, systolic blood pressure, serum cholesterol, TVRS score and BMI, the difference was no longer significant (0.154). Therefore, our overall conclusion is that this study does not support our *first hypothesis* as outlined in Chapter One. In Chapter Three, we learnt how several groups have used the LDI technique to identify impaired microvascular endothelial function in subject groups exposed to the aforementioned cardiovascular risk factors. It is therefore unsurprising that these factors should have a discernible effect on microvascular endothelial function in this group of SSc subjects. Although this study is larger than any previous study of *in-vivo* microvascular endothelial function in SSc, it must be acknowledged that the numbers are still quite low ($n = 15$ per group). As noted previously, the relative rarity of

SSc, together with the rather limited timescale we had, made it difficult to recruit more patients. Perhaps a future study could incorporate even larger study groups, thereby generating a more robust difference between lcSSc and dcSSc.

Chapter Seven

A Study of Brachial Artery and Common Carotid Artery Elasticity In SSc Subgroups vs. Healthy Control

7.1 Introduction

As discussed in Chapter Two, the collagenous ECM of the arterial *tunica media* maintains smooth muscle cell alignment and integrity, while providing a suitable framework in which smooth muscle cells can contract and relax – an essential requirement for the maintenance of normal vessel tone⁹⁷. Crucially, smooth muscle cells are linked to elastic lamellae of the ECM by microfibrils composed of proteins that are encoded by the *fibrillin* gene⁹⁸, so any defects of this protein should interrupt the normal smooth muscle cell/ECM interactions. Any such interruption should be manifested as an alteration of arterial elastic properties; and in fact preliminary evidence for this is provided by Cheng *et al.*, who have demonstrated reduced compliance and increased stiffness in the common carotid arteries of SSc patients^{68;79}.

The *second hypothesis* of this thesis proposes that arterial fibrosis - possibly occurring secondary to fibrillin gene defects - is a widespread phenomenon in SSc. In practical terms this means that arterial elasticity changes should not be confined to large elastic vessels such as the common carotid, but should also be demonstrable in ubiquitous medium and small-sized muscular arteries. Our aim in this chapter was to test this hypothesis, by conducting an investigation to compare the elastic properties of the common carotid artery *and* the brachial artery, in subjects with lcSSc, dcSSc and in healthy controls. The investigation was performed using a duplex ultrasound scanner in conjunction with the previously described Wall Track System software to visualise the arteries and accurately record vessel distension. Elastic properties were calculated using the historically well established algorithms for diametric compliance, Peterson's elastic modulus and stiffness index, as described in Chapter Four.

7.2 Patients and Methods

The study was approved by the local hospital ethics committee according to the principles of the Declaration of Helsinki. Sixty subjects were studied in total, 20 with lcSSc, 20 with dcSSc, and 20 healthy age- and sex-matched healthy controls. Systemic sclerosis was defined according to the criteria of the American College of Rheumatology^{332;388} and was diagnosed by a single senior rheumatologist. All recruited SSc patients had had their condition for at least 2 years. Patients with severe lung disease requiring intra-venous vasodilator or ambulatory oxygen therapy were excluded. Controls were selected from volunteers working in or associated with the Departments of Surgery and Rheumatology. For each subject we recorded age, gender, height and weight (for BMI calculation), and TVRS score. A sample of venous blood was taken for serum cholesterol.

Subjects were examined lying flat on an examination table, and vasoreactive factors were avoided as previously described. Right arm blood pressure was recorded using an automated sphygmomanometer (Johnson and Johnson, UK). All scans were performed by a single trained individual, using a high resolution duplex scanner with a 7.5 MHz linear array probe (Pie 350, Pie Medical Systems, Netherlands), and signal output to a standard personal computer equipped with Wall Track System (WTS) software. Electrocardiograph leads were attached at appropriate sites on the anterior chest wall for ECG-gating of the RF-signal. This equipment setup is described in detail in Chapter Four.

All arterial scans were performed on the right side, with the vessel viewed in the sagittal plane 90° to the long axis. For the common carotid artery, an initial orientating neck scan was performed to identify the internal and external branches, the bulb and the

common carotid itself. WTS scanning was performed at a consistent site immediately proximal to the carotid bulb, at a point clear of atheroma, stenosis and debris, and with well defined arterial walls. The right brachial artery was then identified at a position medial to the biceps tendon and adjacent to the elbow crease. Again, a point clear of atheroma and with well defined arterial walls was chosen for WTS analysis. For each artery three scans were performed per subject, with each scan of 4 seconds duration and returning systolic and diastolic diameters over at least 3 cardiac cycles. The optimum scan, defined as that which had the least variance in systolic/diastolic diameter over the 3 cardiac cycles, was chosen for subsequent analysis.

7.3 Data analysis and statistical methods

The following parameters were used for calculating arterial elasticity : diametric compliance (D), Peterson's elastic modulus (E_p) and stiffness index (β). They were described in detail in Chapter Four, and are summarised in Table 7.1.

The mean systolic and diastolic diameters over the 3 recorded cardiac cycles (the difference between which is the mean distension) is applied to the above equations. As previously stated, 3 cycles were chosen because to record over a longer timeframe would require the subject to be perfectly still for longer periods, and could potentially introduce errors owing to minute drifts in arm position. Values for systolic and diastolic pressure are derived from a single measurement using a standard arm sphygmomanometer after a 10 minute rest period. Basic patient data were compared using one-way ANOVA, with the exception of gender for which a Chi-square test was used. The mean value for each elasticity parameter was compared between groups using one-way ANOVA, and where

significant differences were found a general linear model (GLM univariate) was used to correct for the confounding effects of age, gender, heart rate, systolic blood pressure, serum cholesterol level, BMI and TVRS score. Pearson's correlation co-efficient was employed to test the correlation between elasticity parameters of the brachial artery and the corresponding elasticity parameters of the common carotid artery.

Table 7.1 Arterial Elastic Parameters. Note that Ep is the inverse of D .

Parameter	Equation
Diametrical compliance	$D(mmHg^{-1}) = \frac{(d_s - d_d)/d_d}{(p_s - p_d)}$
Peterson's elastic modulus	$Ep(mmHg) = \frac{(p_s - p_d)}{(d_s - d_d)/d_d}$
Stiffness index	$\beta = \frac{\ln(p_s/p_d)}{(d_s - d_d)/d_d}$

d_s : systolic diameter; d_d : diastolic diameter; p_s : systolic brachial artery pressure; p_d : diastolic brachial artery pressure; \ln : natural log.

Intra-observer variation was calculated as intra-session and inter-session co-efficients of variation. Intra-session variability was calculated for both the carotid and the brachial artery in a subset of 10 subjects, each having 4 scans per artery - giving a total of 12 distension readings per artery per subject. Inter-session variability was calculated in two healthy control subjects, each having 4 scans of the carotid and brachial artery - again giving a total of 12 distension readings per artery per subject - each day for 5 separate days. Although all scans were performed by a single individual, inter-observer variability was calculated in a subset of 5 subjects in whom brachial and carotid artery

scans were performed as normal, after which time a second observer (the vascular technologist responsible for my training) was called in to the room and the measurements were repeated.

7.4 Results

Characteristics of patients and controls are described in Table 7.2. There were no significant differences between groups for age, gender, BMI, serum cholesterol or TVRS score. However, both systolic blood pressure ($p<0.001$) and heart rate ($p=0.013$) were progressively and significantly higher between groups from control to lcSSc to dcSSc.

Systolic and diastolic diameters for both the brachial and common carotid arteries are shown in Table 7.3; there were no significant differences in either systolic or diastolic diameter between groups. The difference between systolic and diastolic diameter - the arterial distension - is also shown in Table 7.3. In the brachial artery, distension was significantly reduced in the dcSSc group compared to both lcSSc and healthy control ($p=0.027$); however, in the common carotid artery there was no significant difference between groups ($p=0.967$).

Results for elasticity parameters are summarised in Table 7.3, and illustrated as vertical scatterplots in Figures 7.1 to 7.6; E_p and β are both directly proportional to arterial elasticity, while D is indirectly proportional. In the brachial artery all three parameters were progressively and significantly impaired from healthy control to lcSSc to dcSSc. Even after correction for the potentially confounding cardiovascular variables, D remained significantly impaired from healthy control to lcSSc to dcSSc ($p<0.001$). In the common carotid artery, all three parameters were progressively and significantly

impaired after correction for confounding variables ($p < 0.001$). Results for correlation of elasticity parameters between brachial artery and carotid artery are shown in Table 7.4.

The results indicate good correlation between these vessels ($p < 0.001$).

Intra-session variability, expressed as a mean co-efficient of variation was 2.4% for brachial artery and 2.7% for carotid artery. Inter-session variability was 3.3% for brachial artery and 3.9% for carotid artery. Inter-observer variability was 5.9% for brachial artery and 6.1% for carotid artery. Intra-session variability for the second observer was assessed in 5 subjects with a total of 12 baseline diameter readings per subject giving a mean co-efficient of variation of 2.7% for brachial artery and 2.5% for carotid artery. Inter-session variability was not assessed for the second observer.

Table 7.2 Subject data. Values are means (SD).

	Healthy controls	lcSSc	dcSSc	<i>p</i> value
Number	20	20	20	
Sex (M/F)	6/14	4/16	4/16	$p = 0.96$
Age	51.6 (12.8)	50.4 (13.2)	53.6 (12.1)	$p = 0.72$
Systolic blood pressure (mmHg)	113.2 (12.9)	127.0 (14.4)	134.8 (13.0)	$p < 0.001$
Diastolic blood pressure (mmHg)	73.8 (9.4)	71.3 (8.7)	70.6 (7.7)	$p = 0.47$
Heart rate	69.9 (9.9)	76.9 (14.5)	81.7 (11.5)	$p = 0.013$
BMI	24.3 (3.0)	25.9 (6.6)	25.4 (3.5)	$p = 0.53$
Serum cholesterol	4.52 (0.9)	5.02 (0.9)	5.1 (1.4)	$p = 0.18$
TVRS score	0.35 (0.7)	0.65 (0.8)	0.85 (0.8)	$p = 0.13$

Table 7.3 Results for brachial and common carotid arteries.

	Healthy controls	lcSSc	dcSSc	Unadjusted <i>p</i> value	Adjusted* <i>p</i> value
Brachial Artery					
Systolic diameter (mm)	4.38 (0.83)	3.92 (0.86)	4.13 (0.79)	0.22	-
Diastolic diameter (mm)	4.22 (0.84)	3.8 (0.84)	4.02 (0.77)	0.28	-
Distension (mm)	1.6 (0.05)	0.11 (0.06)	0.11 (0.08)	0.027	0.148
Ep (mmHg)	1170 (592)	2532 (1940)	4828 (5981)	0.009	0.098
β (arbitrary units)	12.5 (5.67)	26.1 (19.7)	49.6 (62.4)	0.011	0.122
D (mmHg ⁻¹)	10.9 (6.12)	5.57 (3.31)	4.16 (2.75)	<0.001	<0.001
Common carotid artery					
Systolic diameter (mm)	7.10 (1.15)	6.87 (0.87)	7.46 (1.67)	0.349	-
Diastolic diameter (mm)	6.68 (1.16)	6.46 (0.83)	7.06 (1.47)	0.283	-
Distension (mm)	0.41 (0.16)	0.41 (0.13)	0.40 (0.26)	0.967	-
Ep (mmHg)	678 (249)	920 (228)	1371 (606)	<0.001	<0.001
β (arbitrary units)	7.37 (2.55)	9.65 (2.54)	14.05 (6.72)	<0.001	<0.001
D (mmHg ⁻¹)	17.1 (6.94)	11.5 (2.76)	8.60 (3.39)	<0.001	<0.001

*Where one-way ANOVA revealed a statistically significant result ($p < 0.05$), a general linear model (GLM univariate) was applied to incorporate the effect of confounding variables (age, serum cholesterol, BMI and TVRS). Ep = Peterson's elastic modulus, β = stiffness index, D = diametric compliance.

Table 7.4 Correlation of elasticity parameters across groups between the brachial artery and the common carotid artery.

	Pearson's correlation co-efficient	<i>p</i> value
Ep	0.428*	<0.001
β	0.443*	<0.001
D	0.628*	<0.001

*Correlation is significant at the 0.01 level.

Figure 7.1 Peterson's elastic modulus for brachial artery.

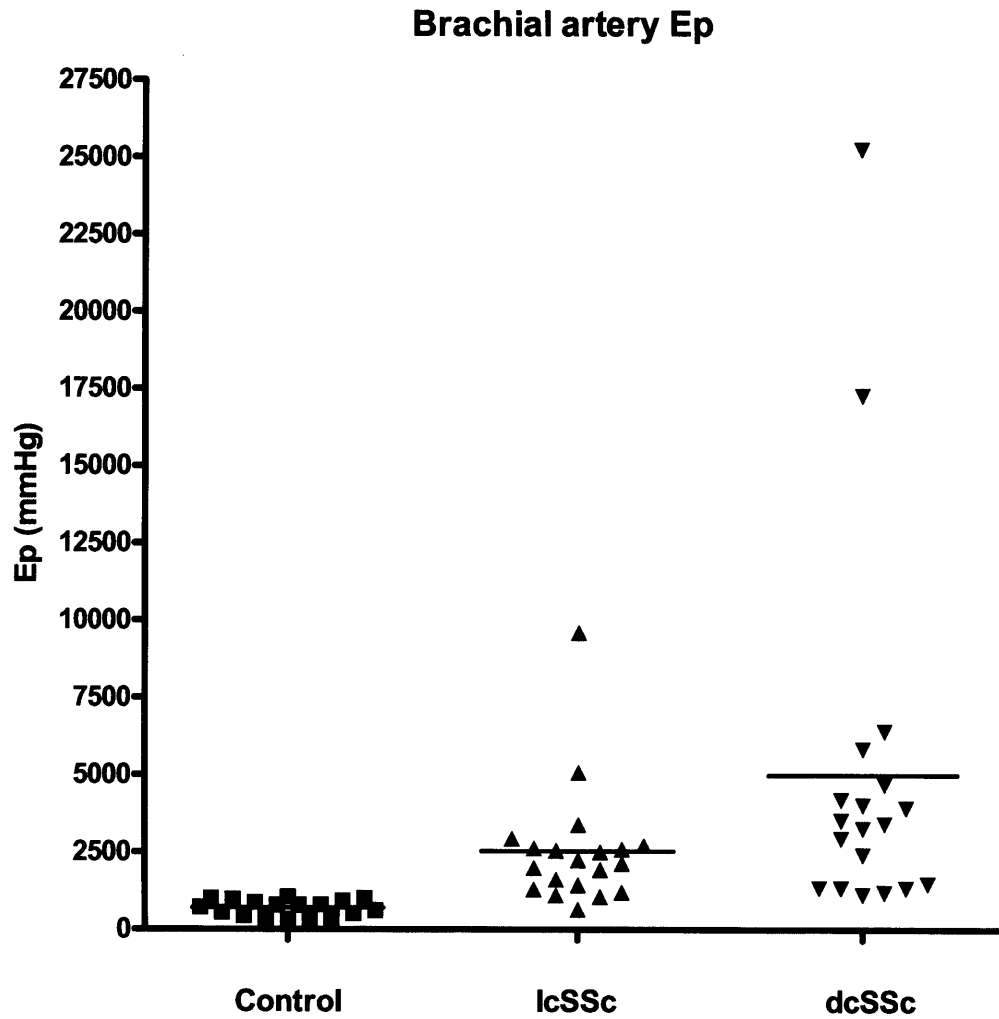


Figure 7.2 Peterson's elastic modulus for carotid artery.

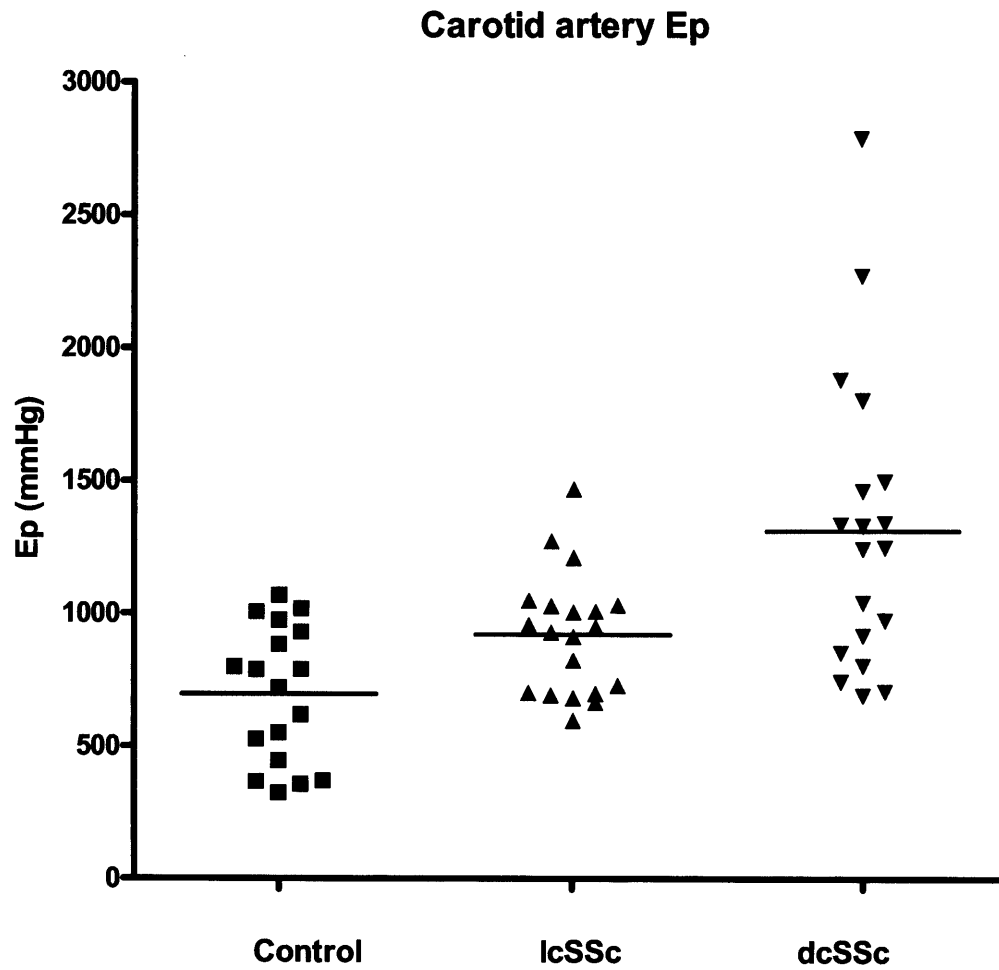


Figure 7.3 Stiffness index for brachial artery.

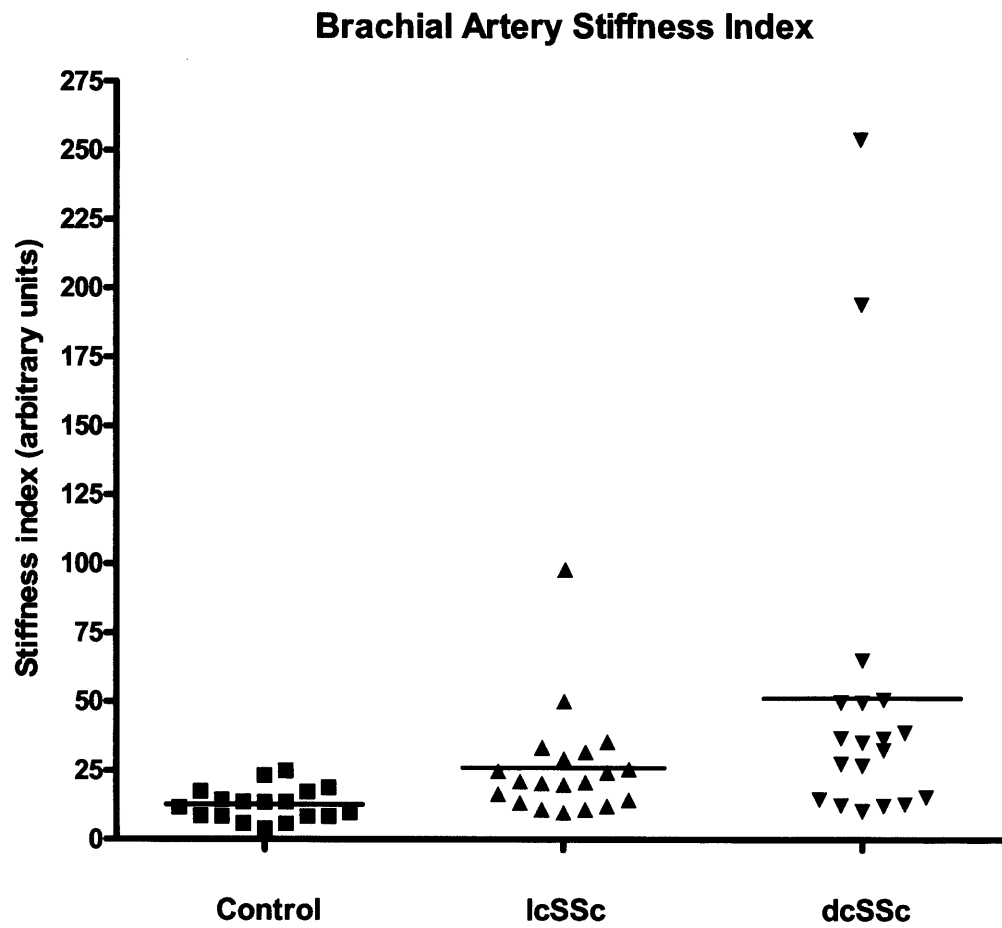


Figure 7.4 Stiffness index for carotid artery.

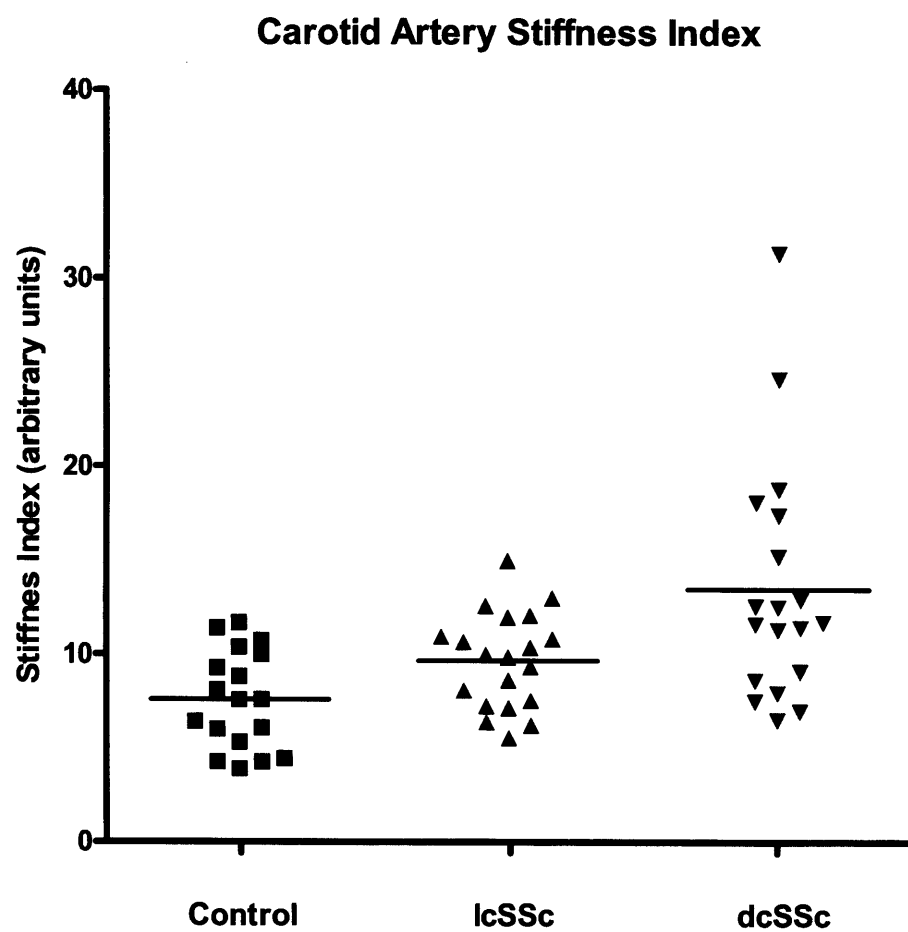


Figure 7.5 Diametric compliance for brachial artery.

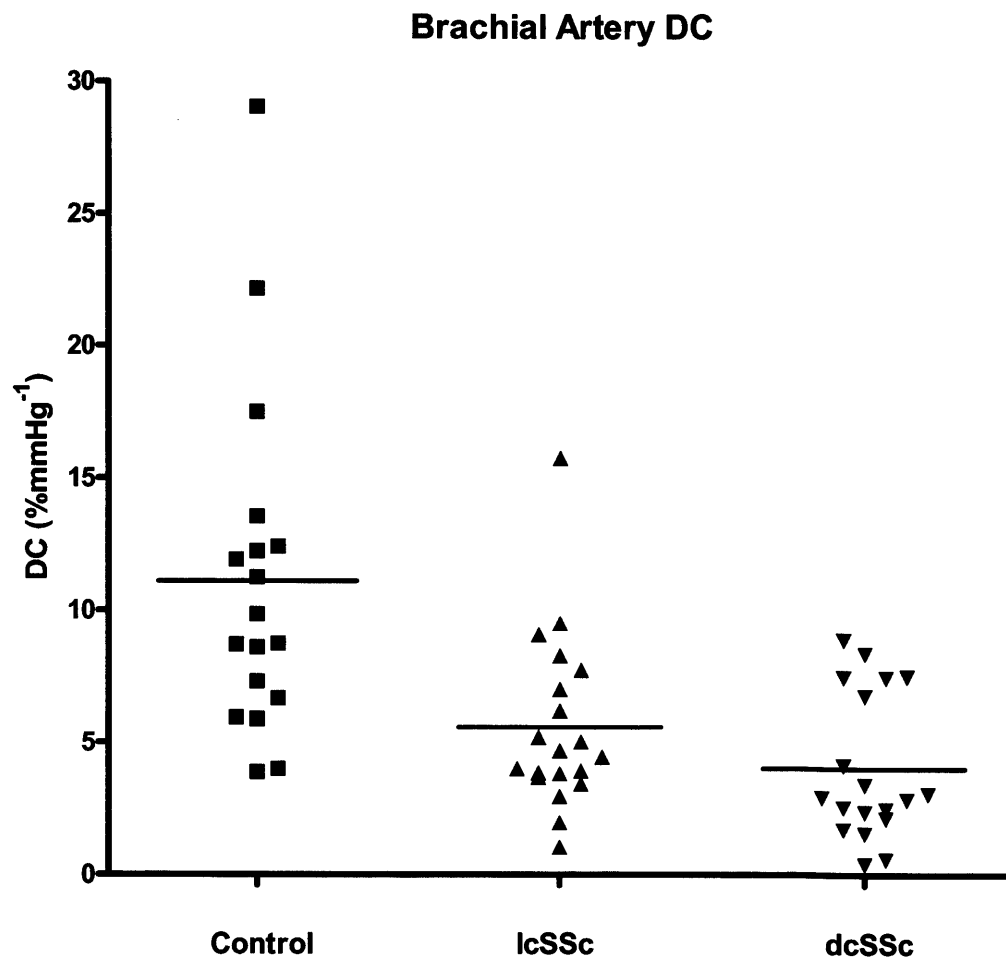
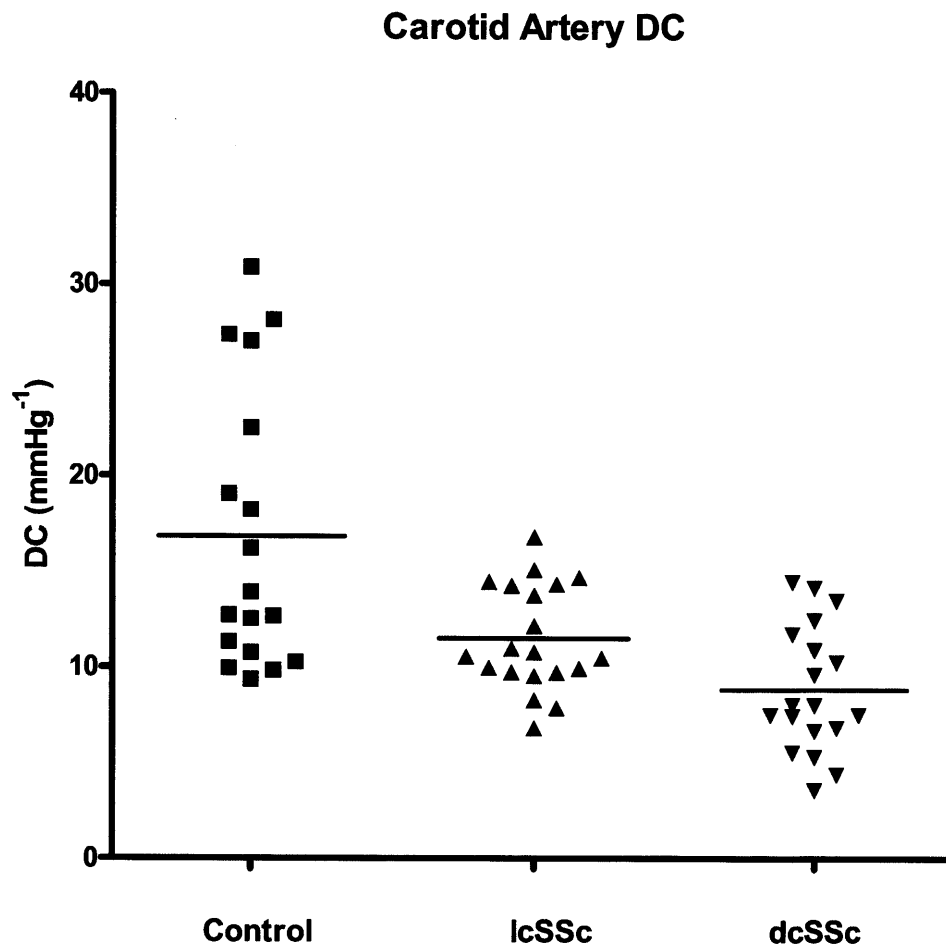


Figure 7.6 Diametric compliance for carotid artery.



7.5 Discussion

The aim for this chapter was to study arterial elastic parameters in the carotid and brachial arteries of SSc subject and controls. Evidence for arterial stiffening was sought *in-vivo* by applying established arterial elasticity parameters, namely Peterson's Elastic Modulus, Diametric Compliance and Stiffness Index. All three parameters have been successfully applied for the study of the common carotid artery, as outlined in Chapter Four. Analysis of the brachial artery is not so commonplace, however, at least two other groups have used a non-invasive echo-locked duplex scanning system similar to ours to apply biomechanical algorithms to this vessel³⁹²⁻³⁹⁴. Bjarnegarde *et al.* applied the distensibility co-efficient and compliance co-efficient (from which equations diametric compliance is derived) in a study of sympathetic stimulation on brachial artery biomechanics³⁹². The same algorithms were applied by Arcaro *et al.* in a study of the effect of acute hyperhomocystinaemia on brachial artery elasticity³⁹⁴. The experiences of these investigators and others provide validation for our methodology.

Our results indicate that common carotid artery elasticity is progressively and significantly impaired from healthy control to lcSSc to dcSSc ($p < 0.001$). This finding was sufficiently robust to withstand correction for cardiovascular risk factors, which are known to adversely affect arterial elasticity in their own right. We also found that brachial artery elasticity was progressively and significantly impaired from healthy control to lcSSc to dcSSc, although the degree of significance was less than that for the common carotid artery ($p = 0.009$, $p = 0.011$ and $p < 0.001$; for Ep, β and D, respectively). However, after correction for cardiovascular risk factors, only diametric compliance remained significantly different between groups ($p < 0.001$).

Our findings in relation to the common carotid artery confirm previous findings by Cheng *et al.* that elasticity of this vessel is impaired in SSc⁶⁸. Since the methodology used in this chapter is almost identical to that used by Cheng *et al.*, this is somewhat reassuring and provides further evidence to validate the use of WTS in this regard. Only one other group has sought to determine arterial compliance in SSc. In 1997, Constans *et al.* conducted a comparative analysis of large vessel distensibility in 18 SSc vs. 18 healthy control subjects, by observing the timing of Korotkoff's sounds³⁹⁵. Using this interesting and non-invasive technique, they found compliance to be significantly reduced in SSc versus control ($p = 0.01$). However, as with other studies conducted around that time, they did not differentiate between SSc subtypes in their study group.

Our findings in relation to the brachial artery are interesting in that only the diametric compliance algorithm was sufficiently robust to withstand correction for cardiovascular risk factors. Diametric compliance is a function of the *relative* change in arterial diameter and is said to facilitate comparisons of vessels of different calibre. Indeed, our decision to incorporate this algorithm was in anticipation of this advantage. Peterson's elastic modulus is the inverse of diametric compliance, and as such the distension algorithm forms the denominator – thus it is less tolerant of relatively small changes in arterial distension, as occurs with the brachial artery.

At first glance, our results seem to suggest that while the latter two algorithms are applicable for the study of large elastic arteries, they are less reliable for medium-sized muscular vessels such as the brachial artery. A more likely interpretation, however, is that they *are* applicable for this calibre of vessel, but that our results are explained by the simple fact that brachial artery elasticity was impaired to a lesser degree than the carotid

artery, and therefore more susceptible to correction for the effect cardiovascular risk factors. In this scenario, diametric compliance would appear to be a more sensitive marker for brachial artery elasticity, in line with our expectations before the study. In any case, diametric compliance was progressively and significantly impaired in our study groups, and this leads us to the conclusion that SSc subjects *do* manifest evidence of arterial fibrosis in their brachial arteries. We do acknowledge, however, that more work needs to be done to study the applicability of these algorithms to arteries of varying calibre and composition.

SSc is primarily a disease of excessive fibrosis and uncontrolled collagen deposition caused by biosynthetically activated fibroblasts. As highlighted in Chapter Two, the mechanisms by which this fibrosis occurs are complex, intertwined, and still incompletely understood. In recent times, the concept of a genetic mutation as a critical step leading to excessive systemic fibrosis in SSc, has been gaining ground. Several investigators have identified mutations of the gene that encodes fibrillin, a fundamental component protein of extra-cellular matrix (ECM), in patients with SSc. Under normal circumstances, the collagenous ECM of the tunica media maintains smooth muscle cell alignment and integrity, while providing a suitable framework in which smooth muscle cells can contract and relax – an essential requirement for the maintenance of normal vessel tone⁹⁷. It is theorised that an abnormal and unstable fibrillin protein causes the inappropriate activation of cellular repair mechanisms, and that this ultimately results in uncontrolled collagen deposition. The *second hypothesis* of this thesis argues that in the presence of underlying genetic abnormalities in SSc, arterial elasticity parameters should

be impaired in multiple vessel types, and not merely confined to large “elastic” vessels such as the common carotid, as suggested in previous studies.

Our findings would seem to support this hypothesis, but only to a degree. Our results provide evidence that brachial artery elasticity is impaired, but to a lesser extent than carotid artery elasticity. When one considers that the fibrillin protein has a major role in the regulation of elastin fibre assembly, this discrepancy between brachial and carotid is perhaps not surprising. While elastin is a crucial component of much of the vascular tree, it is expressed to a greater extent in the walls of large central arteries such as the carotid^{396,397}. However, the fact that brachial artery elasticity is also impaired, provides further confirmation of the system-wide nature of the fibrotic reaction, and lends weight to the concept of an underlying genetic abnormality as a possible cause.

Chapter Eight

An Assessment of Common Carotid Viscoelasticity in SSc Subgroups vs. Healthy Control

8.1 Introduction

In the previous chapter, biomechanical algorithms (E_p , β and D) were used for the assessment of arterial compliance in SSc patients. Although this is regarded as an acceptable means of assessing vessel elasticity, they are relatively static algorithms because they rely on single systolic and diastolic pressure/diameter measurements for calculation³⁸³. Given the nature of arterial walls, such one-off measurements, although useful, do not provide an adequate reflection of how diameter responds to changing pressure. As discussed in Chapter Four, the arterial wall is composed of a complex arrangement of layers, and in disease processes such as SSc, each layer can be affected to a greater or lesser extent. This complexity ensures that the relationship between pressure and diameter is non-linear, and when one is plotted against the other the result is a curve, known as a *hysteresis loop*³⁸⁵. The biomechanical impact of disease processes on arterial walls are manifested as alterations in the nature of this loop. Focusing our attention on the pressure/diameter relationship will therefore provide a dynamic representation of the properties of the arterial wall (*viscoelasticity*), and in theory this should provide an accurate reflection of how disease processes alter wall movement^{382;383}.

Non-invasive *in-vivo* measurement of intra-luminal pressure at the precise site of diameter measurement requires the use of a technique known as *applanation tonometry*. This was used in conjunction with the previously described ultrasound scanner and WTS software. This equipment setup allows precise measurement of diameter, as it changes with changing intra-luminal pressure within the arterial segment under study. A mathematical algorithm was then applied to the resulting hysteresis loop to determine the *phase angle*, which is a reflection of the viscoelastic properties of the arterial wall^{384;385}.

In Chapter Seven, we provided evidence of reduced brachial and carotid arterial elasticity in dcSSc and lcSSc compared to control, suggesting that the fibrotic reaction known to affect the skin and internal organs of these patients, also involves the arterial tree. However, thus far no group has sought to determine *viscoelastic* properties in SSc. In the *second hypothesis* of this thesis, we propose that arterial viscoelastic properties are impaired in SSc. Therefore, the aim for this chapter was to measure diameter change in response to intra-luminal pressure change, and to investigate whether the phase angle calculated from the derived hysteresis loops varied between healthy control, lcSSc and dcSSc groups.

8.2 Patients and Methods

The study was approved by the local hospital ethics committee according to the principles of the Declaration of Helsinki. Patients were selected according to the criteria previously described. Forty subjects were studied in total, 13 with lcSSc, 14 with dcSSc, and 13 age- and sex-matched healthy controls. As before, basic patient data was recorded and care was taken to avoid potentially vasoreactive factors.

Subjects were examined lying supine. Right arm blood pressure was recorded using the automated sphygmomanometer used in previous chapters. All scans were performed by a single trained individual, using the previously described duplex scanner, and Wall Track System software. All carotid arterial scans were performed on the right side, with the vessel viewed in the sagittal plane 90° to the long axis. After identifying the point of maximal pulsation, an initial scan was performed to ensure that this site was free of atheroma and had well-defined arterial walls. An applanation tonometry probe (Millar

Instruments Inc) was then applied to the skin overlying this point, and adhered in place using dressing tape. The ultrasound probe was placed immediately upstream of the tonometry probe and WTS scanning commenced. During WTS scanning gentle pressure was applied to the tonometry probe so as to generate a good pressure signal. This methodology is described in detail in Chapter Four.

8.3 Data analysis and statistical methods

For each artery three scans were performed per subject, with each scan of 4 seconds duration. Change in diameter and pressure was recorded over at least 3 cardiac cycles per scan. The optimum scan, defined as that which had the least variance in systolic/diastolic diameter and pressure over the 3 cardiac cycles, and displayed the greatest wave amplitude, was chosen for subsequent analysis.

The data was downloaded onto a spreadsheet and we then performed intra-luminal pressure calibration, hysteresis loop generation and application of the phase angle algorithm, as described in detail in Chapter Four. Intra-session and inter-session variability for carotid artery diameter and pressure was calculated as previously described, and expressed as mean co-efficients of variation. Inter-observer variability was not assessed. Statistical analyses were performed by comparing means using one-way ANOVA, and a general linear model was applied to correct for confounding variables.

8.4 Results

Basic patient data are shown in Table 8.1. Groups are well matched for most parameters, however, in-line with findings in previous chapters, there was a progressive and

significant difference in heart rate and systolic blood pressure from healthy control to lcSSc to dcSSc.

Hysteresis loops for healthy control, lcSSc and dcSSc are shown in Figures 8.1, 8.2 and 8.3 respectively. Table 8.4A provides an illustration of how mean diameter increases as mean pressure increases (from 70mmHg to 130mmHg), in control, lcSSc and dcSSc. Table 8.4B provides similar information, however, in this graph the *change* in diameter is plotted against increasing pressure. This graph provides a clear illustration of how as pressure increases (particularly from 70mmHg to 80mmHg), diameter increases more sharply in healthy controls compared to lcSSc and dcSSc.

Results for phase angle are shown in Table 8.2, and illustrated as a vertical scatter plot in Figure 8.5. Phase angle was markedly less in dcSSc compared to lcSSc and control, but this finding was marginally outside the significance level ($p = 0.053$). After correction for potentially confounding cardiovascular risk factors this increased to $p = 0.164$. Intra-session variability, expressed as a mean co-efficient of variation was 2.7% for diameter and 2.8% for pressure. Inter-session variability was 3.9% for diameter and 7.1% for pressure.

Table 8.1 Basic subject data. Values are means (SD).

	Healthy control	lcSSc	dcSSc	<i>p</i> value
Number	13	13	14	
Sex (M/F)	9/13	10/13	11/14	<i>p</i> = 0.97
Age	54.7 (12.1)	52.2 (13.4)	51.3 (13.2)	<i>p</i> = 0.78
Systolic blood pressure (mmHg)	112.5 (12.2)	127.1 (12.7)	131.1 (11.9)	<i>p</i> < 0.01
Diastolic blood pressure (mmHg)	76.7 (9.4)	71 (9.1)	68.7 (7.0)	<i>p</i> = 0.06
Heart rate	68.8 (8.6)	77.6 (14.7)	83.4 (11.2)	<i>p</i> < 0.01
BMI	24.2 (3.4)	23.6 (2.3)	25.1 (3.6)	<i>p</i> = 0.47
Serum cholesterol	4.7 (1.0)	5.0 (1.0)	4.9 (1.6)	<i>p</i> = 0.75
TVRS score	0.46 (0.8)	0.69 (0.86)	0.71 (0.73)	<i>p</i> = 0.66

Table 8.2 Results for phase angle. Values are means (SD).

	healthy control	lcSSc	dcSSc	<i>p</i> value	<i>p</i> value*
phase angle	21.1° (6.2)	19.2° (6.8)	14.7° (7.2)	<i>p</i> = 0.053	<i>p</i> = 0.164

* This *p* value is corrected for the potentially confounding cardiovascular risk factors age, heart rate, systolic blood pressure, BMI, TVRS score and serum cholesterol.

Figure 8.1 Hysteresis loops for control subjects (n=13).

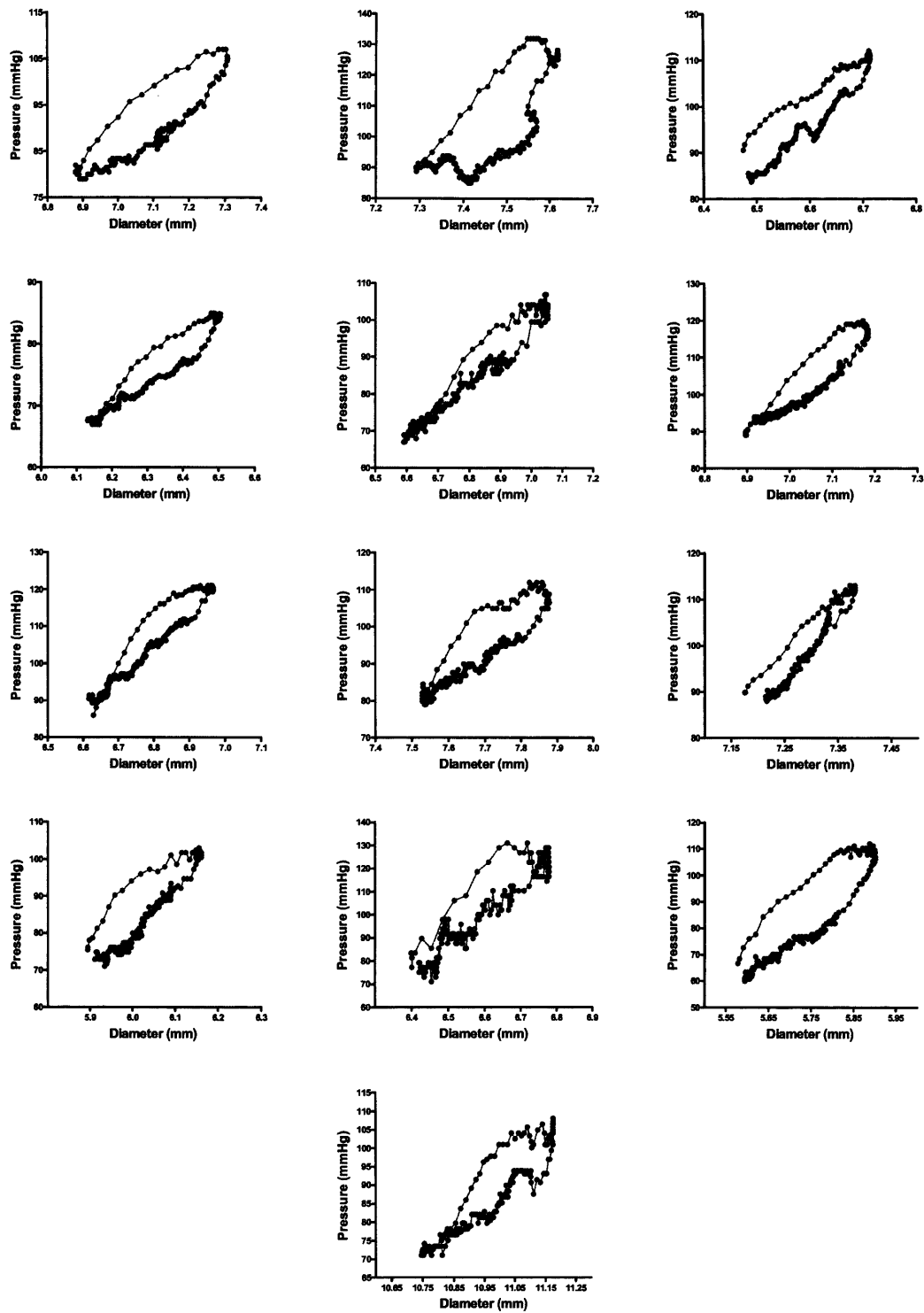


Figure 8.2 Hysteresis loops for lcSSc subjects (n=13)

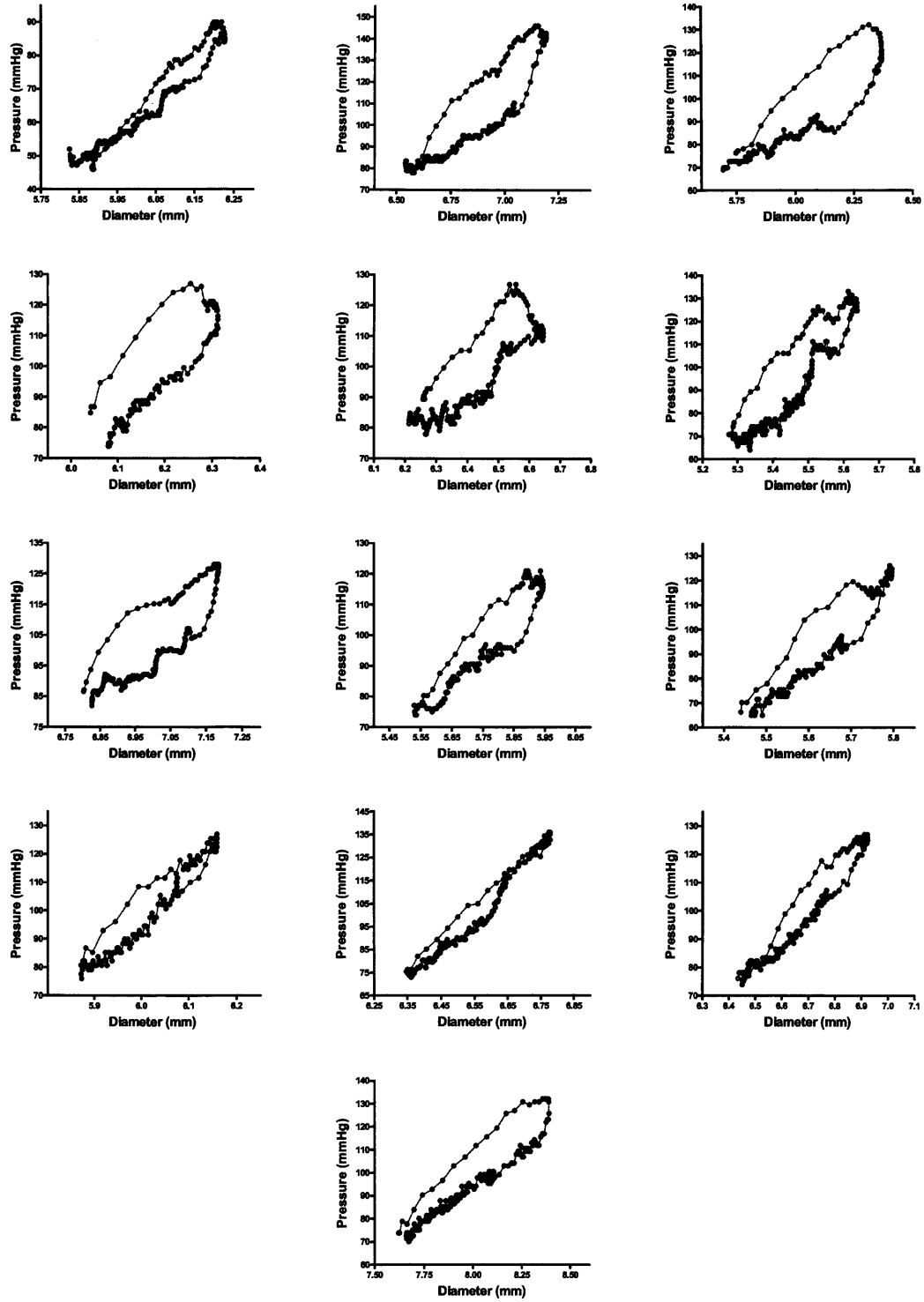


Figure 8.3 Hysteresis loops for dcSSc subjects (n=14).

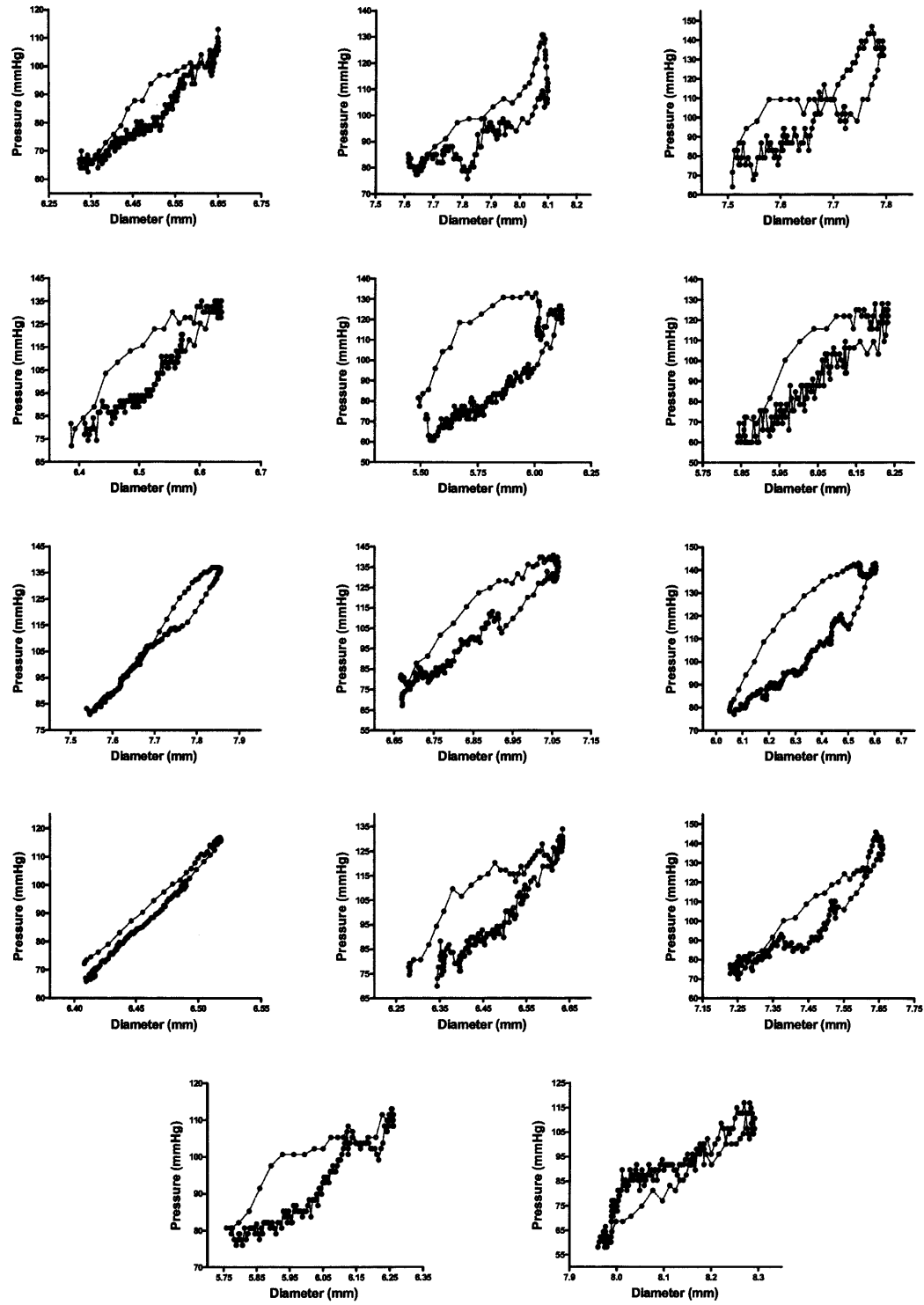


Figure 8.4A Mean (SD) arterial diameter at a given pressure (over the range 70 to 130mmHg) in control, lcSSc and dcSSc groups.

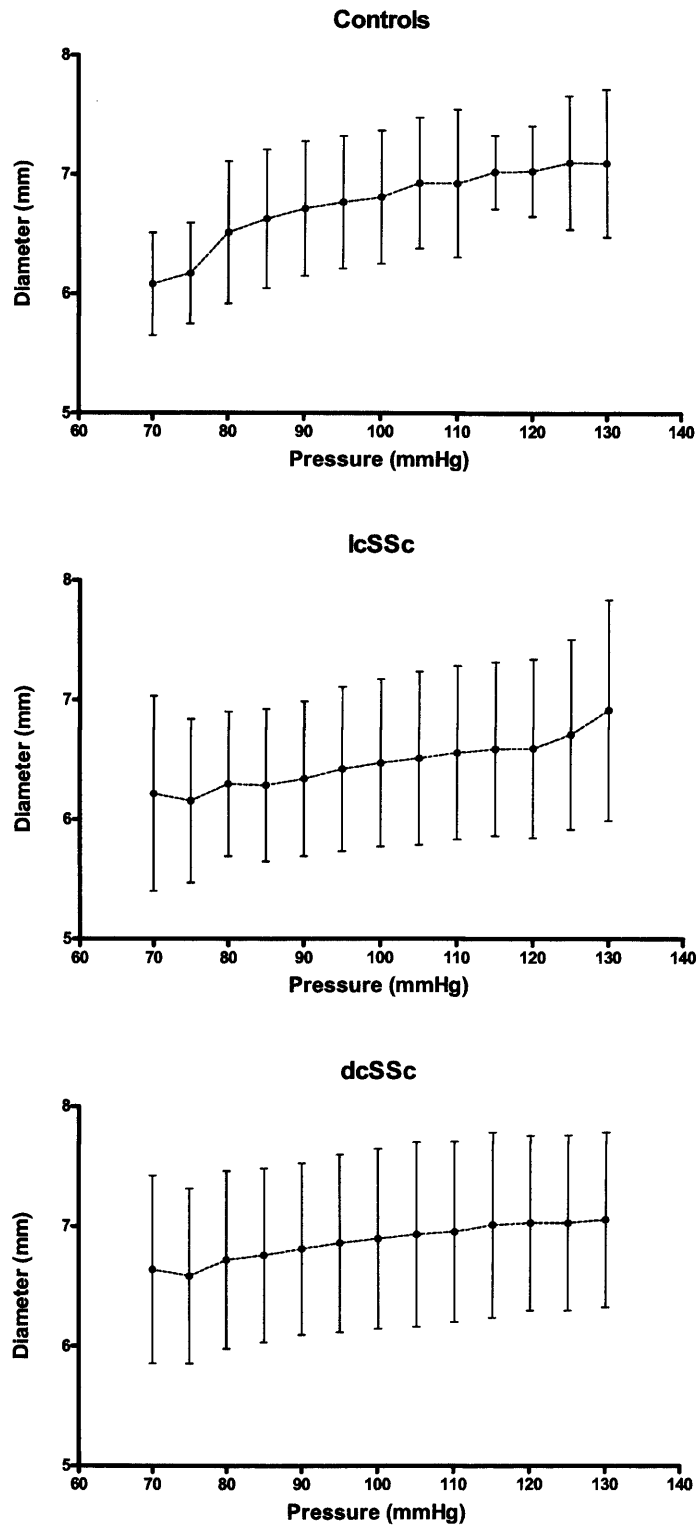


Figure 8.4B *Change in mean arterial diameter for a given pressure (over the range 70 to 130mmHg) between control, lcSSc and dcSSc groups.*

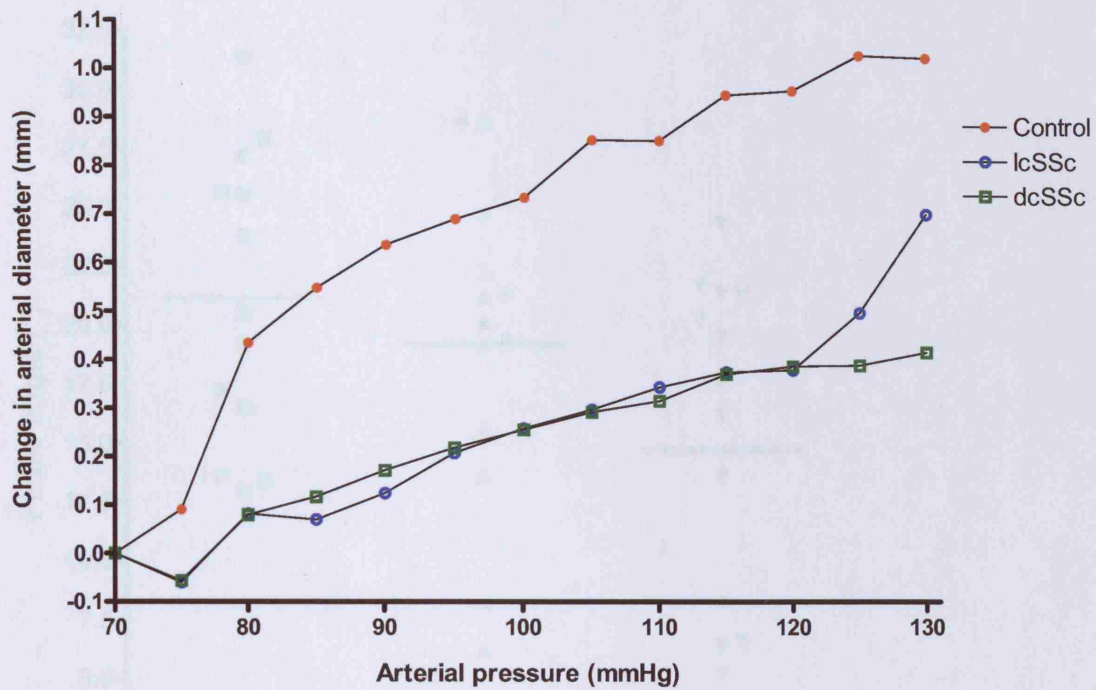
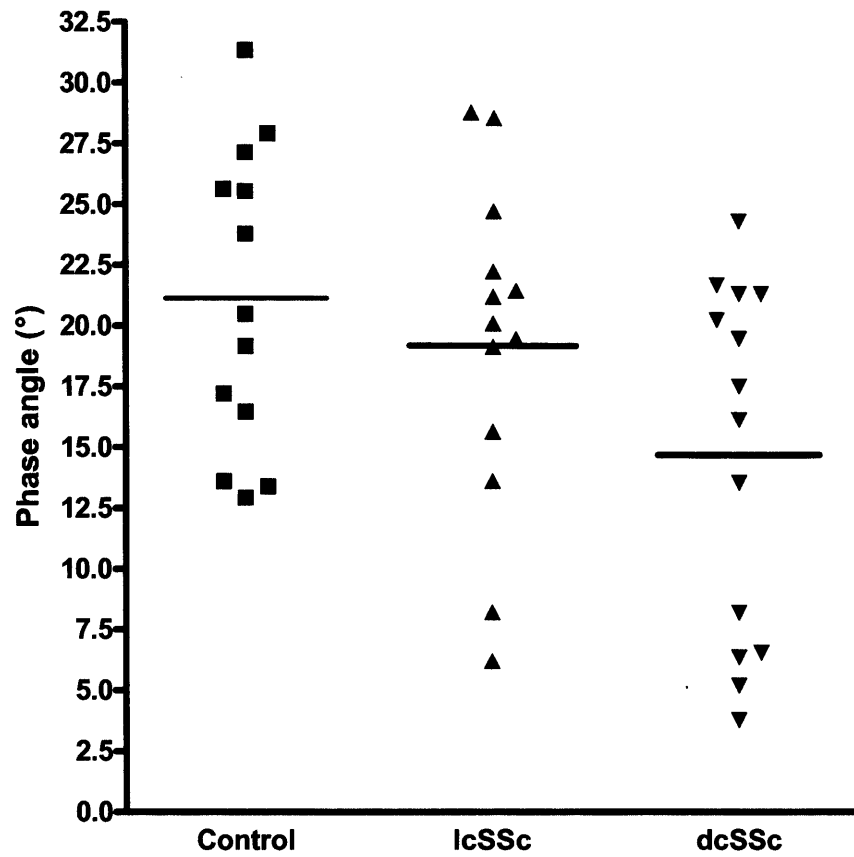


Figure 8.5 Vertical scatter plot demonstrating the difference in mean phase angle between healthy controls, lcSSc and dcSSc groups.



8.5 Discussion

Biomechanical properties of arteries have been studied for decades, but it is only in recent years that sufficient technological advances have been made to enable accurate characterisation of the dynamic motion of arterial walls. The two central technical advances relate to echo-locked wall tracking systems, and to applanation tonometry. This technology has been applied and validated for the *in-vivo* assessment of arterial viscoelastic properties in healthy subjects³⁸³, and in conditions such as hypertension³⁸². However, ours is the first study to apply this technology for the investigation of *in-vivo* arterial viscoelastic properties in SSc.

Applanation tonometry is an exciting step forward in our quest to learn more about arterial function. Not only is the procedure non-invasive, it is easy to perform and the equipment is relatively inexpensive. But there are certain limitations to its use. As discussed in Chapter Four, currently available tonometer probes only allow assessment of relatively superficial arteries such as the common carotid and radial artery. Gentle manual pressure must be applied such that the probe slightly flattens (or applanates) the arterial segment under study. If there is a sizeable thickness of intervening tissue between arterial wall and skin, the signal denoting arterial pulsation will dissipate during transfer to the probe, creating a potential source of error when studying such conditions as obesity and diabetes. However, in our experience with SSc subjects this effect appears to be negligible, and this is probably a reflection of the relatively low BMI of these groups.

Focusing solely on superficial vessels goes some way to overcoming a related problem, namely the application of excessive hold-down pressure in order to achieve a good intra-luminal pressure signal. Only gentle pressure should be applied to the artery,

because otherwise the biomechanical properties of the arterial segment under study will become distorted. Such distortion will be manifested as a reduction in the amplitude, or irregularities of the pressure signal. In our study, great care was taken to avoid applying excessive hold-down pressure, however, it must be acknowledged that there is a certain amount of subjectivity involved in this aspect of the technique. Despite having a thin study population, ultimately the degree of hold-down force being applied is a function of the observer and his or her appreciation of potential technical problems. To minimise this problem, all measurements were performed by a single observer (myself), and only clear pressure signals with good amplitude were used for analysis. Our figures for intra- and inter-session variability for uncalibrated intra-luminal pressure provide reassurance that the pressure signal was of good quality, and was an accurate and reproducible reflection of intra-luminal pressure over repeated measurements.

Accurate arterial diameter measurement is also important for hysteresis loop generation, and this was accomplished using the previously described echo-locked duplex ultrasound scanner with edge-detection (WTS) software. For this study, it was important to precisely correlate diameter with intra-luminal pressure, as measured by the tonometry probe, for each recorded data point in the cardiac cycle (at a sampling rate of 200Hz). Previous studies have relied on *non*-contemporaneous measurements, whereby diameter is measured over several cardiac cycles, after which pressure is measured at the same point over several more cardiac cycles, and the data points are subsequently merged using a spreadsheet^{382;383}. Such systems are inherently more prone to error, and may have an adverse effect on the quality of the derived hysteresis loops. Given that calculation of viscoelastic properties requires application of one or more mathematical algorithms,

which in turn are reliant on good quality hysteresis loops, it is of paramount importance that the derived loops are as accurate as possible. In our study, WTS software allowed us to measure diameter and intra-luminal pressure contemporaneously over the *same* cardiac cycles, and these were plotted against each other for each individual data point to generate the pressure curve. We feel that this adaptation to the previously established methodology increases the accuracy of the derived hysteresis loops, and as far as we are aware this is the first time this approach has been applied for an *in-vivo* study.

We have already shown that elasticity equations, such as diametric compliance, are impaired in SSc subsets. However, as stated above, a particular advantage of this technique is that it allows a dynamic representation of arterial wall motion as intra-luminal pressure increases and decreases, and this is demonstrated in Figures 8.4A and 8.4B. These graphs show how, for a given increase in intra-luminal pressure, diameter increases more readily in healthy controls compared to both lcSSc and dcSSc, thus providing further confirmation of reduced arterial compliance in these groups.

We found phase angle to be lower in both dcSSc and lcSSc compared to healthy control, but this finding was just outside the significance range ($p = 0.053$). When a general linear model was applied to correct for the potentially confounding effect of age, heart rate, systolic blood pressure, BMI, serum cholesterol and TVRS score, the difference in phase angle between groups was reduced ($p = 0.164$). In light of our findings in previous chapters, it is not surprising that correction for these cardiovascular risk factors should affect the significance of the result, and indeed systolic blood pressure has already been shown to have an independent adverse effect on arterial viscoelasticity³⁸². It is somewhat disappointing that our findings are just outside the

significance range, but while it must be acknowledged that this could mean that there really is no difference in viscoelasticity between groups, we do however feel that there is evidence of a trend whereby viscoelastic properties are impaired in both dcSSc and lcSSc compared to healthy controls. Clearly, however, this impairment has not been demonstrated to the same extent as that seen for the purely elastic parameters applied in Chapter Seven, and perhaps this is a reflection of the smaller study populations that were used for viscoelasticity assessment ($n = 13$), compared to elasticity assessment ($n = 20$).

In Chapter Seven, we found evidence that arterial elasticity is progressively and significantly impaired from healthy control to lcSSc to dcSSc, and this is in keeping with the *second hypothesis* of this thesis. Part of this hypothesis also states that arterial viscoelastic properties are impaired in lcSSc and dcSSc versus control. Although our findings would appear to provide some support for this assertion, ultimately the hypothesis must be rejected because the data is outside the significance range. However, our study does provide a novel *in-vivo* demonstration of viscoelastic properties in SSc subjects, and will hopefully pave the way for future work using this technology.

Chapter Nine

Conclusion

9.1 Introduction

Systemic sclerosis has provided medical practitioners with a diagnostic and therapeutic challenge for centuries. In modern times, rheumatologists provide the lead in diagnosing and treating these patients, however, the multi-system nature of the disease ensures that healthcare providers across various specialties are involved in their care, from cardiologists and nephrologists to physiotherapists and specialist nurses. Much of the morbidity and mortality of SSc is now known to arise as a direct result of pathological processes that target the blood vessels in various organ systems, and therefore the condition has specifically attracted the attention of vascular surgeons and others with an interest in vascular disease.

An in-depth literature review of the pathophysiology of the condition (Chapter Two) revealed the daunting and bewildering array of possible mechanisms by which damage to blood vessels may be mediated. To a certain extent a lack of knowledge as to the precise nature of these pathological processes, and more importantly how they interact with each other, was exposed. With this in mind, it was critical that from the outset, the aims of this thesis were clearly defined and that only achievable goals were set. At an early stage, it became apparent that the study could be broadly and conveniently categorised into *endothelial dysfunction* and *altered arterial biomechanics*, and accordingly two hypotheses were devised. This approach was deemed necessary in order to provide a coherent *structure* to the thesis, otherwise the sheer complexity of the various theories underpinning vascular injury in SSc would threaten to make the subsequent analysis somewhat chaotic. At the same time, the emerging difference between lcSSc and dcSSc provides the thesis with a degree of *narrative* which was not

anticipated when we embarked on the study. Throughout the thesis there is evidence, some highly significant and some just outside the significance range, that vascular injury is more pronounced in the dcSSc subset.

9.2 Summary of findings

9.2a First hypothesis

There is much evidence in the literature, emerging primarily from *in-vitro* studies, to suggest that endothelial dysfunction is a key feature of the disease process. Given the relative lack of *in-vivo* work in this area, we contended as our *first hypothesis* that endothelial dysfunction could be demonstrated *in-vivo* in SSc patients. We also hypothesised that the two major disease subtypes manifested endothelial dysfunction to differing degrees, reflecting their differing clinical profiles.

We then conducted a thorough critical review of the literature to learn about and identify the best available methods for non-invasive *in-vivo* assessment of endothelial function (Chapter Three). From this review, two particular methods were chosen for inclusion. Firstly, the data collection phase of the study commenced with an assessment of brachial artery flow-mediated dilatation in groups of lcSSc, dcSSc and healthy controls (Chapter Five). This study demonstrated for the first time statistically significant impairment of non-microvascular endothelial function in the dcSSc group compared to both lcSSc and healthy controls.

There then followed an assessment of microvascular endothelial function using laser Doppler iontophoresis (Chapter Six). For this study, we concentrated solely on the differences between lcSSc and dcSSc, and did not include a healthy control group. This

was because other investigators have already made comparisons between SSc and healthy control groups^{389;390}, and based on our findings in Chapter Five we decided to conduct a focused comparative analysis of lcSSc versus dcSSc. From this study we were able to conclude that microvascular endothelial function was not significantly impaired in dcSSc compared to lcSSc, although there was evidence of a trend in this direction.

Our first hypothesis therefore appears to be correct when using the FMD technique, but is not proven when using LDI. We accept that our LDI study had certain failings, particularly in relation to study size, and also because of the lack of a healthy control group, and that these failings will have to be addressed in future studies.

9.2b Second hypothesis

SSc is primarily a disease of excessive fibrosis that mainly appears to affect the skin and solid internal organs. Following our review of vascular function in SSc (Chapter Two), it became apparent that similar processes could affect the systemic vascular tree, and if so this would be manifested as reduced elasticity and altered arterial biomechanics. As our *second hypothesis*, we contended that this was indeed the case, and further that such changes would be system-wide and therefore manifested in arteries other than the common carotid artery, as had previously been demonstrated. We also hypothesised that changes would be apparent when assessing dynamic (viscous) properties as well as static (elastic) properties.

We set about testing this hypothesis on the common carotid and brachial arteries in lcSSc, dcSSc and healthy control groups, by accurate non-invasive measurement of arterial distension and pressure, and applying established mathematical algorithms to

calculate vessel compliance, stiffness and elasticity (Chapter Seven). We confirmed previous findings in relation to the common carotid artery that these parameters are progressively impaired from dcSSc to lcSSc to healthy control groups⁶⁸. But we were also able to provide a novel demonstration of statistically significant impairment of compliance in the brachial arteries of the dcSSc group.

Demonstration of arterial viscous properties required a different approach (Chapter Eight). For this we used an applanation tonometer probe to accurately and non-invasively measure intra-luminal pressure of the common carotid artery, and concurrently measured diameter at the same site, thereby recording the change in diameter in real-time as pressure changed during the cardiac cycle. This was plotted to give hysteresis loops, from which the phase angle was derived using a mathematical algorithm. Although we found phase angle to be lower in the dcSSc groups, suggesting a degree of correlation with findings in Chapter Seven, this was marginally outside the significance range, and therefore, not in keeping with our hypothesis. Nevertheless, this was the first time this novel technique has been demonstrated in SSc patients, and we believe it has enormous value as a non-invasive investigative tool for assessing *in-vivo* arterial biomechanics in this and other conditions.

9.3 SSc – What have we learnt?

Without doubt, a fundamental question for any investigator is “what have we learnt from our study?” (Table 9.1). We feel that we have addressed several interesting and important issues relating to vascular function in SSc, that appear to have been thus far inadequately

Table 9.1. Main learning points.

Significant finding	Method used	Scientific implication	Potential clinical implication
dcSSc subset demonstrates greater endothelial dysfunction.	Flow mediated dilatation	Confirms the widely-held view that endothelial injury, leading to imbalances of mediators such as NO and ET-1, is a component of vascular dysfunction.	Possible therapeutic implications, eg. targeting and monitoring use of bosentan or iloprost.
dcSSc subset demonstrates altered arterial biomechanics.	Arterial elasticity parameters	Suggests structural changes in arterial smooth muscle, which supports the idea of arterial fibrosis as a cause of vascular dysfunction.	Possible implications for diagnosis and disease staging (easy, well tolerated and non-invasive test).
Vascular impairment is demonstrable at more than one site, suggesting system-wide involvement.	Arterial elasticity parameters (carotid and brachial); brachial artery FMD; microvascular involvement (Ref).	Supports the idea of system-wide arterial injury, either with a genetic cause (eg. fibrillin defects), or with an acquired defect in cytokine and/or endothelial cell activity.	Perhaps disease detection at one site could predict disease at another (eg. correlation between carotid elasticity and incidence of renal crisis).

addressed in the literature. Furthermore, our findings in a couple of areas in particular, could challenge established opinion on vascular disease in this condition.

First, an interesting and unexpected trend that emerged from our study was the finding that the dcSSc subgroup manifested a greater degree of vascular dysfunction than lcSSc, and this was apparent in both of the study's hypotheses (the aforementioned *narrative*). From our review in Chapter Two, it was clear that while much work has been done to study vascular function in SSc, investigators tended not to place any great distinction between the SSc subgroups. This is probably best explained by the fact that in some parts of the world the large majority of cases fall into the lcSSc category, and therefore meaningful subdivisions become difficult to achieve. However, it is clear from our work that there are significant differences in vascular function between these two groups. Perhaps it is therefore time for all investigators of this condition, particularly those involved in laboratory based research, to change their population selection methodology to reflect these differences.

This point leads to an obvious follow-on question, which is *why* the dcSSc subgroup should manifest greater vascular dysfunction. Our literature review in Chapter Two did not yield a clear answer to this question, and as stated this is likely to be because most laboratory investigators do not specifically study vascular function in the dcSSc subgroup. In fact digging a little deeper, and introducing a clinician's perspective, it becomes apparent that many practising rheumatologists regard the lcSSc subset as being "traditionally" the more active group in terms of vascular disease. The evidence for this assumption is anecdotal at best, and is no doubt based on the observation that these patients typically have long-standing Raynaud's disease and present more often with

vascular complications of this condition. But this belief does reflect a prevailing view among both clinicians and scientists, that SSc vascular disease solely, or at least primarily, affects the microvasculature.

We believe our study provides a challenge to this dogma, and we feel that it is time to accept the reality that vascular dysfunction, both in regard to endothelial cell activity and arterial smooth muscle function, is not confined to the microcirculation, but can be demonstrated in the arterial walls of larger arteries. Further, our findings validate the use of endothelial cells sourced from larger vessels for laboratory research. Although there is as yet no clear biochemical answer as to *why* the dcSSc subset in particular should manifest greater vascular dysfunction, perhaps this is related to site of injury, with larger vessels being more readily involved in dcSSc.

So following on from this, the second issue that we feel our study has highlighted is the *system-wide* nature of vascular dysfunction. With regard to arterial biomechanics, we have identified abnormalities in both medium-sized muscular arteries and large elastic vessels. The notion of SSc being a systemic disease with widespread tissue fibrosis and collagen deposition is not new, and several theories exist as to the mechanisms behind this phenomenon. In recent times, much interest has rested on an underlying genetic abnormality, specifically involving the fibrillin gene, a theory that has been discussed in detail in Chapter Two. Such defects would provide an explanation as to why elasticity changes are observed in the brachial as well as the carotid arteries, but it is not necessarily the only explanation. Chapter Two also highlighted evidence of acquired defects in signalling pathways involved in collagen synthesis, specifically involving cytokines such as TGF- β . It is not yet clear how fibrillin gene defects and cytokine

abnormalities fit together in disease evolution, but each in isolation, or both together, could have system-wide effects which are manifested as altered arterial biomechanics.

With regard to endothelial dysfunction, our findings in the brachial artery, together with data from other groups suggesting microvascular and even pulmonary artery endothelial dysfunction, lends weight to this also being a system-wide phenomenon. Potential causes of endothelial cell injury are discussed in Chapter Two, and given the ubiquity of endothelial cells throughout the vascular tree, it is relatively easy to envisage an injurious agent having a system-wide effect. What that agent is, and how it interacts with known defects affecting cellular fibrosis, are critical questions that remain to be answered.

9.4 Clinical Correlations

Correlating our findings directly to current clinical practice is difficult, but we feel that our experience could point the way for others to look at how vascular function relates directly to clinical features of SSc. However, a couple of important caveats should be applied before too many grand conclusions regarding clinical usefulness are drawn.

We must first and foremost acknowledge the limitations of our study with regard to clinical profiling of patients. From the outset, we strove simply to compare lcSSc with dcSSc, and with healthy controls. Our initial aim was to concentrate on the vascular techniques, striving towards methodological water tightness. We did not introduce complex parameters, such as presence of specific vascular complications or varying disease duration, into our selection criteria because to do so would have made the project difficult to achieve in terms of adequate patients numbers, in the timeframe that we had.

Another important caveat is that our methodology, although widely used as research tools, has yet to be validated for clinical and diagnostic use. Several investigators have observed that while these methods are useful for group comparisons in a research environment, they lack the robustness that would be required for looking at individuals or even small groups^{203;218;224}.

Nevertheless, there are at least two areas worth highlighting where our findings could be valuable to future investigators and clinicians (Table 9.1). First, in the diagnosis and staging of the condition, particularly in relation to assigning to subgroups. At present, diagnosis is based on clinical evidence gathered during the initial and subsequent examinations, and although this process has been formally structured into criteria endorsed by the ACR³³², one has to acknowledge the subjectivity of this approach. Although diagnostic adjuncts, such as antibody profiling and nailfold capillaroscopy, can be of assistance, they lack sufficient sensitivity and specificity to serve as routine diagnostic tests. Non-invasive tests of vascular function, such as those used in our thesis, could prove useful in differentiating lcSSc from dcSSc, and the degree of vascular dysfunction could give an indication of disease severity or stage.

Second, with regard to predicting vascular or non-vascular complications, and targeting therapeutic agents accordingly. Both subgroups of this condition can cause vascular complications such as erectile dysfunction, critical digital ischaemia and ulceration, pulmonary hypertension and hypertensive renal crisis^{19;29}. In addition, there are suggestions that these patients could have a higher risk of cardiac disease compared to the population mean²⁹. Perhaps our methodology could prove useful in identifying which groups are most susceptible to these complications, for example, by correlating carotid

artery elasticity or brachial artery FMD to renal crisis or pulmonary hypertension. Subsequently, drug treatments such as bosentan and iloprost could be delivered accordingly.

9.5 Future directions

We have identified at least three areas that we feel need to be addressed as a result of our findings. First, as highlighted in the section above, it is imperative that our methodology is re-applied to groups of SSc patients, but with a strong emphasis on clinical features such as disease duration and presence of vascular complications. As we have acknowledged, our work aimed to look at various aspects of vascular function in SSc subgroups, and did not delve into specific clinical areas in much detail. Areas that could be looked at are effect of disease duration on vascular function, effect of drug treatments, presence of one or other of the numerous complications, co-existence of coronary artery disease and comparisons with other related conditions such as systemic lupus erythematosus and primary Raynaud's disease. Incorporating these parameters will require a study on a much larger scale involving widely varying patient groups, and ideally concentrating solely on one method, thereby shifting the focus away from different measurement techniques and towards the clinical features of systemic sclerosis.

Second, it would be interesting to see if the trends we identified, particularly in relation to LDI and hysteresis loops where marginally non-significant vascular impairment was evident in the dcSSc group, are of any potential significance. Once again this would require a study that would be larger in scope, perhaps using a sole method in a larger patient group.

And third, all investigators, particularly those involved in laboratory work, should devote more time into looking at subgroups of SSc. Our findings suggest that it is no longer acceptable to design studies looking broadly at “SSc versus control”, as there is simply too much variation within the parent group. Our findings also provide a validation for the use of endothelial cells sourced from larger vessels in such studies.

When embarking on this study, we primarily wanted to explore techniques for non-invasive *in-vivo* assessment of endothelial function and arterial biomechanics. The choice of which subject population we should apply our techniques to came somewhat later. After much reading and investigation, SSc became the obvious choice. Here was a condition that provided vascular investigators such as ourselves, with a fascinating “test-bed” in which to demonstrate abnormal vascular function *in-vivo*. Furthermore, many aspects of this debilitating condition still remain a mystery, fuelling the need for further research. We consider ourselves fortunate that we had access to a large population of such patients, who without exception were ready and willing to help with our investigation, often at their own expense in terms of time and convenience. There is much work that still needs to be done in this field, and we hope that our experience will prove useful to future researchers.

Publications and Presentations

Assessment of brachial artery endothelial function in scleroderma using non-invasive ultrasound.

T.A. Alam, R. Santhiripala, C. P. Denton, C. M. Black, A. Seifalian, D. Baker.
Presented to the Society of Academic and Research Surgery, Belfast, Jan 2004.

Assessment of brachial artery elasticity in scleroderma using non-invasive ultrasound.

T.A. Alam, R. Santhiripala, C. P. Denton, C. M. Black, A. Seifalian, D. Baker.
Presented to the Society of Academic and Research Surgery, Belfast, Jan 2004.

Different subgroups of scleroderma show different patterns of vascular involvement, as assessed by brachial artery FMD.

T.A. Alam, R. Santhiripala, C. P. Denton, C. M. Black, G. Hamilton, D. Baker, A. Seifalian.
Presented to the International Society for Applied Cardiovascular Biology, Savannah, USA, March 2004.

Brachial artery elasticity is impaired in the diffuse subset of scleroderma.

T.A. Alam, R. Santhiripala, C. P. Denton, C. M. Black, G. Hamilton, D. Baker, A. Seifalian.
Presented to the International Society for Applied Cardiovascular Biology, Savannah, USA, March 2004.

A review of methods currently used for assessment of in vivo endothelial function.

T. A. Alam, A. M. Seifalian, D. Baker.
Eur J Vasc Endovasc Surg. 2005 Mar;29(3):269-76. Review.

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